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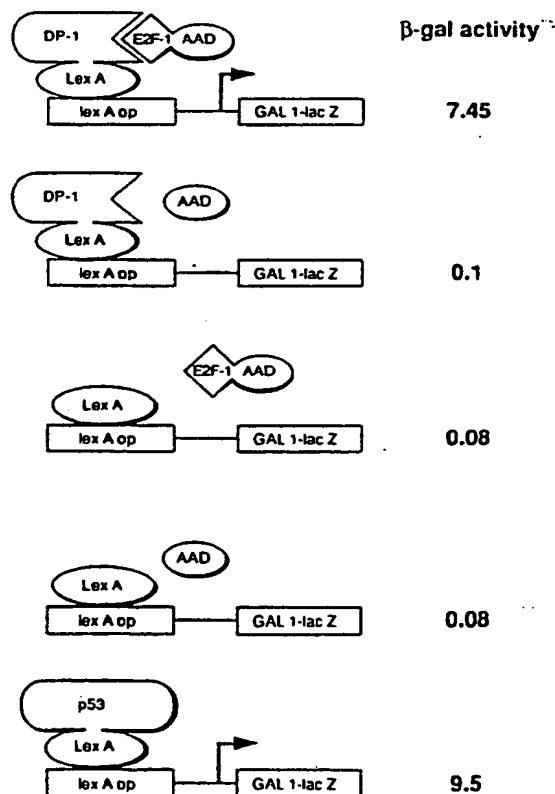
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(54) Title: TRANSCRIPTION FACTOR DP-1

(57) Abstract

The invention provides a polynucleotide in substantially isolated form which comprises a contiguous sequence of nucleotides which is capable of selectively hybridizing to Seq. ID No. 1 or to the complement of Seq. ID No. 1, and a polypeptide in substantially isolated form which comprises: (a) the protein of Seq. ID No. 2; or (b) an allelic variant or species homologue thereof; or c) a protein at least 70 % homologous to (a); or (d) a fragment of any one of (a) to (c) capable of forming a complex with the E2F-1 protein or related family member; or (e) a fragment of any one of (a) to (c) of at least 15 amino acids.



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TRANSCRIPTION FACTOR DP-1

This invention relates to a novel transcription factor, to its
5 production and uses.

The molecular events that occur during the cell cycle need to be integrated with the transcription apparatus so that gene expression can be synchronised with cell cycle progression.

10 Recently, a transcription factor called DRTF1 or E2F has been identified and shown to bind to pRb, the protein product of the retinoblastoma susceptibility gene, an anti-oncogene or tumour suppressor gene (see for example Wagner and Green, Nature 352, 189-190, 1991). It is widely believed that the cellular
15 transcription factor DRTF1/E2F functions as a key component in cell cycle control because it associates with important cell cycle regulating proteins, such as the retinoblastoma gene product (pRb), p107, cyclins and cyclin-dependent kinases, and furthermore its transcriptional activity is modulated by certain
20 viral oncoproteins, such as adenovirus Ela, SV40 large T antigen, and the human papilloma virus E7 protein.

It is believed that the transcription factor DRTF1/E2F plays an important role in integrating cell cycle events with the
25 transcription apparatus because, during cell cycle progression in mammalian cells, it undergoes a series of periodic interactions with molecules that are known to be important regulators of cellular proliferation. For example, the retinoblastoma tumour suppressor gene product (pRb), which negatively regulates
30 progression from G1 into S phase and is frequently modified in tumour cells binds to DRTF1/E2F. Similarly, the pRb-related protein p107 occurs predominantly in an S phase complex with DRTF1/E2F. Both pRb and p107 repress the transcriptional activity of DRTF1/E2F, which is likely to be fundamentally
35 important for regulating cellular proliferation because DRTF1/E2F binding sites (the E2F site) occur in the control regions of a variety of genes that are involved with proliferation, such as c-myc and p34^{cdc2}. Furthermore, mutant Rb proteins, encoded by alleles isolated from tumour cells, fail to bind to DRTF1/E2F,

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and hence are unable to interfere with E2F site-dependent transcriptional activation. Another important feature of DRTF1/E2F is that certain viral oncoproteins, such as adenovirus Ela, SV40 large T antigen and human papilloma virus E7, modulate its activity by sequestering pRb and p107 from the inactive transcription factor. This effect requires regions in these viral proteins that are necessary for transformation of tissue culture cells and hence to overcome growth control. Thus, the ability of these oncoproteins to regulate DRTF1/E2F may be the means by which they over-ride the normal mechanisms of cellular growth control and, conversely, transcriptional repression by pRb may be the basis of pRb-mediated negative growth control.

A potential mechanism for integrating the transcription-regulating properties of pRb and p107 with other cell cycle events was suggested by the identification of cyclin A and the cdc2-related cyclin-dependent kinase p33^{cdk2} in the DRTF1/E2F complex. Cyclin A is necessary for progression through S phase, a function that could perhaps be mediated through its ability to recruit the cyclin-dependent kinase p33^{cdk2} to DRTF1/E2F. Taken together these data suggest that DRTF1/E2F is a transcription factor whose primary role may be to relay cell cycle events to the transcription apparatus via molecules such as pRb, p107, cyclins and cdks, thus ensuring that gene expression is synchronised and integrated with cell cycle progression.

More recently, a transcription factor with the properties of E2F has been cloned and sequenced (Helin et al, Cell 70 (1992), 337-350 and Kaelin et al, Cell 70 (1992), 351-364).

30

We have now surprisingly found that the protein termed E2F is a complex of factors, comprising the factor cloned by Helin et al and Kaelin et al, and a novel protein, whose cDNA sequence is presented below as Seq. ID No. 2. The sequence of the cDNA encoding this protein is shown below as Seq. ID No. 1. The new protein is referred to by us as DP-1. While not wishing to be bound by any one particular theory, it is believed that the factor cloned by Helin et al and Kaelin et al may dimerise with DP-1. Evidence presented in the accompanying examples shows that

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DP-1 and the protein of Helin et al (referred to as E2F-1) form a complex which is involved in the regulation of transcription.

5 It has also been found that E2F-1 is one of a family of related transcription factor components. Members of this family are believed to interact with DP-1 to form a series of above transcription factors.

10 Brief description of the drawings.

Figure 1 shows the results of affinity purification of the DP-1 protein from F9 EC cells.

15 Figure 2 shows DP-1 is a DNA binding polypeptide in the E2F complex.

Figure 3 shows sequence specific DNA binding of DP-1.

20 Figure 4 shows a comparison of DP-1 and E2F-1.

Figure 5 summarises the constructs used and results obtained in experiments showing DP-1 activates E2F site dependent transcription in vivo.

25

Figure 6 shows that DP-1 and E2F-1 exist in the same protein complex in vivo.

Figure 7 shows that DP-1 and E2F-1 bind to the E2F site as a
30 complex.

Figure 8 shows the interaction of DP-1 and E2F-1 in yeast cells.

Figure 9 shows there is functional synergy between DP-1 and E2F-1
35 in Drosophila cells.

Figure 10 shows DP-1 contributes to E2F site-dependent transcription in F9 EC cells.

Figure 11 shows DP-1 and E2F-1 activate E2F site-dependent transcription in yeast cells.

The present invention thus provides a polynucleotide in substantially isolated form which comprises a contiguous sequence of nucleotides which is capable of selectively hybridizing to Seq. ID. No 1 or to the complement of Seq. ID No. 1. A polynucleotide of the invention includes a DNA of Seq. ID No. 1, and fragments thereof capable of selectively hybridizing to Seq. ID No. 1. A further embodiment of the invention provides a DNA coding for the protein of Seq. ID No. 2 or a fragment thereof.

The polynucleotide may also comprise RNA. It may also be a polynucleotide which includes within it synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the oligonucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the in vivo activity or lifespan of oligonucleotides of the invention used in methods of therapy.

A polynucleotide capable of selectively hybridizing to the DNA of Seq. ID No. 1 will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the DNA of Seq. ID No. 1 over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Such a polynucleotide will be referred to below as a polynucleotide according to the invention.

A polynucleotide of the invention will be in substantially isolated form if it is in a form in which it is free of other polynucleotides with which it may be associated in the natural environment of the body. It will be understood that the polynucleotide may be mixed with carriers or diluents which will

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not interfere with the intended purpose of the polynucleotide and still be regarded as substantially isolated.

A polynucleotide according to the invention may be used to
5 produce a primer, e.g. a PCR primer, a probe e.g. labelled with
a revealing label by conventional means using radioactive or non-
radioactive labels, or the polynucleotide may be cloned into a
vector. Such primers, probes and other fragments of the DNA of
Seq. ID No. 1 will be at least 15, preferably at least 20, for
10 example at least 25, 30 or 40 nucleotides in length, and are also
encompassed by the term "a polynucleotide according to the
invention" as used herein.

A polynucleotide such as a DNA polynucleotide according to the
15 invention may be produced recombinantly, synthetically, or by any
means available to those of skill in the art. It may be also
cloned by reference to the techniques disclosed herein.

The invention further provides a double stranded polynucleotide
20 comprising a polynucleotide according to the invention and its
complement.

A further embodiment of the invention provides vectors for the
replication and expression of a polynucleotide, in particular a
25 DNA or RNA polynucleotide, according to the invention. The
vectors may be, for example, plasmid, virus or phage vectors
provided with an origin of replication, optionally a promoter for
the expression of the said polynucleotide and optionally a
regulator of the promoter. The vector may contain one or more
30 selectable marker genes, for example an ampicillin resistance
gene in the case of a bacterial plasmid or a neomycin resistance
gene for a mammalian vector. The vector may be used in vitro,
for example for the production of RNA or used to transfect or
transform a host cell. The vector may also be adapted to be used
35 in vivo, for example in a method of gene therapy.

A further embodiment of the invention provides host cells
transformed or transfected with the vectors for the replication
and expression of a polynucleotide according to the invention,

including the DNA Seq. ID No. 1 or the open reading frame thereof. The cells will be chosen to be compatible with the vector and may for example be bacterial, yeast, insect or mammalian.

5

A polynucleotide according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by
10 synthetic means. Such antisense polynucleotides may be used in a method of controlling the levels of the protein of Seq. ID No. 2 in a cell. Such a method will include the step of introducing into the cell the antisense polynucleotide in an amount effective to inhibit or reduce the level of translation of the DP-1 mRNA
15 into protein. The cell may be a cell which is proliferating in an uncontrolled manner such as a tumour cell.

The invention further provides a protein of Seq. ID. No. 2, homologues thereof, and fragments of the sequence and its
20 homologues, which is capable of functioning as a mammalian transcription factor. In particular, the invention provides a polypeptide in substantially isolated form which comprises:

- (a) the protein of Seq. ID No. 2; or
- (b) an allelic variant or species homologue thereof; or
- 25 (c) a protein at least 70% homologous to (a); or
- (d) a fragment of any one of (a) to (c) capable of forming a complex with the E2F-1 protein or related family member; or
- (e) a fragment of any one of (a) to (c) of at least 15
30 amino acids.

All polypeptides within this definition are referred to below as a polypeptide according to the invention.

35 A polypeptide of the invention will be in substantially isolated form if it is in a form in which it is free of other polypeptides with which it may be associated in the natural environment of the body. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the

intended purpose of the polynucleotide and still be regarded as substantially isolated.

A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, eg. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

10 An allelic variant of a polypeptide of the invention will be a variant which will occur naturally in a murine animal and which will function to regulate gene expression in a substantially similar manner to the protein of Seq. ID No. 2. Similarly, a species homologue of the protein will be the equivalent protein
15 which occurs naturally in another species, including man, and which performs the equivalent function in that species to the DP-1 protein of Seq. ID No. 2 in murine animals. Within any one species, a homologue may exist as several allelic variants, and these will all be considered homologues of the Seq. ID No. 2
20 protein. Allelic variants and species homologues can be obtained by following the procedures described herein for the production of the protein of Seq. ID No. 2 and performing such procedures on a suitable cell source, eg from a rodent carrying an allelic variant or another species. Since the protein appears to be
25 evolutionarily conserved it will also be possible to use a polynucleotide of the invention to probe libraries made from rodent or other cells in order to obtain clones encoding the allelic or species variants. The clones can be manipulated by conventional techniques to identify a polypeptide of the
30 invention which can then be produced by recombinant or synthetic techniques known per se. Preferred species homologues include mammalian or amphibian species homologues.

A protein at least 70% homologous to the Seq. ID No. 2 will be
35 preferably at least 80 or 90% and more preferably at least 95% homologous to the protein of Seq. ID No. 2 over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by

those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

- 5 Generally, fragments of Seq. ID No. 2 or its allelic variants or species homologues thereof capable of forming a complex with the E2F-1 protein will be at least 10, preferably at least 15, for example at least 20, 25, 30, 40, 50 or 60 amino acids in length.
- 10 It will be possible to determine whether fragments form a complex with the E2F-1 protein by providing the E2F-1 protein and the fragment under conditions in which the E2F-1 protein and DP-1 normally form a trans-activating transcription factor, and determining whether or not a complex has formed. The
- 15 determination may be made by, for example, measuring the ability of the complex to bind an E2F binding site *in vitro*, or alternatively, determining the molecular weight of the putative complex by methods such as SDS-PAGE.
- 20 Preferred fragments include those which are capable of forming a trans-activation complex with E2F-1 or its related family members. The examples herein describe a number of methods to analyse the function of the DP-1 protein and these may be adapted to assess whether or not a polypeptide is capable of forming a
- 25 trans-activation complex with the E2F-1 protein. For example, the fragment can be added to E2F-1 in the presence of a reporter gene construct adapted to be activated by the DP-1/E2F-1 complex, as described in Figure 10. Such an experiment will determine whether the polypeptide fragment has the necessary activity.
- 30 A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I , enzymes, antibodies and linkers such as
- 35 biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of DP1 protein in a sample.

A polypeptide or labelled polypeptide according to the invention may also be fixed to a solid phase, for example the wall of an immunoassay dish.

5 The present invention also provides polynucleotides encoding polypeptides of the invention, for example double-stranded DNA polynucleotides. Such polynucleotides can be incorporated into a recombinant replicable vector. The vector may be used to replicate the DNA. Preferably, the DNA in the vector is operably
10 linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably
15 linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences. Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention.

20

Thus, in a further aspect the invention provides a process for preparing a polypeptide according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to
25 provide for expression by the vector of a coding sequence encoding the polypeptide, and recovering the expressed polypeptide.

The invention also provides monoclonal or polyclonal antibodies
30 to a polypeptide according to the invention. The invention further provides a process for the production of monoclonal or polyclonal antibodies to the protein of Seq. ID No. 2 or to a polypeptide of the invention. Monoclonal antibodies may be prepared by conventional hybridoma technology using the proteins
35 or peptide fragments thereof, as an immunogen. Polyclonal antibodies may also be prepared by conventional means which comprise inoculating a host animal, for example a rat or a rabbit, with a peptide of the invention and recovering immune serum.

Fragments of monoclonal antibodies according to the invention which retain their antigen binding activity, such Fv, F(ab') and F(ab₂)' fragments form a further aspect of the invention. In addition, monoclonal antibodies according to the invention may be
5 analyzed (eg. by DNA sequence analysis of the genes expressing such antibodies) and humanized antibody with complementarity determining regions of an antibody according to the invention may be made, for example in accordance with the methods disclosed in EP-A-0239400 (Winter).

10

The present invention also provides compositions comprising a polypeptide of the invention together with a carrier or diluent. Such compositions include pharmaceutical compositions in which case the carrier or diluent will be pharmaceutically acceptable.

15

The present invention further provides compositions comprising an antibody or fragment thereof of the invention together with a carrier or diluent. Such compositions include pharmaceutical compositions in which case the carrier or diluent will be
20 pharmaceutically acceptable.

Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral
25 (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active
30 ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

35

For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the

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intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the polypeptide to blood components or one or 5 more organs.

Peptides according to the invention, antibodies or fragments thereof to peptides according to the invention and the above-mentioned compositions may be used for the treatment, regulation 10 or diagnosis of conditions, including proliferative diseases, in a mammal including man. Such conditions include those associated with abnormal (eg at an unusually high or low level) and/or aberrant (eg due to a mutation in the gene sequence) expression of one or more transcription factors such as the E2F factor 15 cloned by Helin et al or the protein of Seq. ID No. 2 or the E2F-1 protein or related family members. The conditions also include those which are brought about by abnormal expression of a gene whose gene product is regulated by the protein of Seq. ID No. 2. Treatment or regulation of conditions with the above-mentioned 20 peptides, antibodies, fragments thereof and compositions will usually involve administering to a recipient, in need of such treatment an effective amount of a polypeptide, antibody, fragment thereof or composition.

25 One group of preferred polypeptides according to the invention are those which are based upon the region of amino acids 160-220 of Seq. ID No. 2. This region of the protein has a homology of about 40% to a similar region of the E2F-1 protein described by Helin et al (ibid) and both regions are putative alpha-helical 30 regions. While not wishing to be bound by any one particular theory, we believe that the heterodimerisation of E2F and the protein according to the invention is mediated through these homologous regions. Accordingly, a preferred embodiment of the invention is to polypeptides of the invention based upon this 35 region, and pharmaceutical compositions containing such polypeptides, for use in a method of inhibiting the activation of transcription factors via the disruption of the formation of the E2F-1-Seq. ID. No. 2 protein complex.

The invention also provides antibodies, and fragments thereof, targeted to this region of Seq. ID No. 2 in order to achieve the above described effect.

5 The present invention further provides an immunoassay for detecting the presence or absence of a polypeptide of the invention in a sample which comprises:

- (a) providing an antibody according to the invention;
- 10 (b) incubating the sample with said antibody under conditions that allow for the formation of an antibody-antigen complex; and
- (c) detecting said antibody-antigen complex.

15 In another aspect, the invention provides a novel assay for identifying putative chemotherapeutic agents for the treatment of proliferative or viral disease which comprises bringing into contact E2F-1 protein or a derivative thereof a polypeptide of the invention and a putative chemotherapeutic agent, and
20 measuring the degree of inhibition of formation of the E2F-1-Seq. ID. No. 2 protein complex caused by the agent. It may not be necessary to use complete E2F-1 and/or Seq. ID. No. 2 protein in the assay, as long as sufficient of each protein is provided such that under the conditions of the assay in the absence of agent,
25 they form a heterodimer. Thus, the invention provides a screening method for identifying putative chemotherapeutic agents for the treatment of proliferative disease which comprises

(A) bringing into contact:

- (i) a polypeptide according to the invention,
- 30 (ii) (a) the E2F-1 protein, or
- (b) an allelic variant or species homologue thereof, or
- (c) an E2F-1 family member having at least 70% homology over the DNA binding region, or
- 35 (d) a fragment of (a), (b) or (c) capable of forming a functional trans-activation complex with the protein of Seq. ID No. 2; or
- (e) a fusion protein comprising (a), (b), (c), or (d); and

- (iii) a putative chemotherapeutic agent;
under conditions in which the components (i) and (ii) in the
absence of (iii) form a complex, and
(E) measuring the extent to which component (iii) is able to
5 disrupt said complex. In the assay, any one or more of the three
components may be labelled, eg with a radioactive or colorimetric
label, to allow measurement of the result of the assay. Putative
chemotherapeutic agents include peptides of the invention.
- 10 Variants, homologues and fragments of E2F-1 protein are defined
in a corresponding manner to the variants, homologues and
fragments of the DP-1 protein.

The complex of (i) and (ii) may be measured, for example, by its
15 ability to bind an E2F DNA binding site in vitro. Alternatively,
the assay may be an in vivo assay in which the ability of the
complex to activate a promoter comprising an E2F binding site
linked to a reporter gene is measured. The in vivo assay may be
performed for example by reference to the examples which show
20 such an assay in yeast, insect, amphibian or mammalian cells.

Candidate therapeutic agents which may be measured by the assay
include fragments of 10 or more amino acids of

- (a) the protein of Seq. ID No. 2
25 (b) an allelic variant or species homologue thereof; or
(c) a protein at least 70% homologous to (a).

Vectors carrying a polynucleotide according to the invention or
a nucleic acid encoding a polypeptide according to the invention
30 may be used in a method of gene therapy. Such gene therapy may
be used to treat uncontrolled proliferation of cells, for example
a tumour cell. Methods of gene therapy include delivering to a
cell in a patient in need of treatment an effective amount of a
vector capable of expressing in the cell either an antisense
35 polynucleotide of the invention in order to inhibit or reduce the
translation of DP-1 mRNA into DP-1 protein or a polypeptide which
interferes with the binding of DP-1 to E2F-1 or a related family
member.

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The vector is suitably a viral vector. The viral vector may be any suitable vector available in the art for targeting tumour cells. For example, Huber et al (Proc. Natl. Acad. Sci. USA (1991) 88, 8039) report the use of amphotrophic retroviruses for the transformation of hepatoma, breast, colon or skin cells. Culver et al (Science (1992) 256; 1550-1552) also describe the use of retroviral vectors in virus-directed enzyme prodrug therapy, as do Ram et al (Cancer Research (1993) 53; 83-88). Englehardt et al (Nature Genetics (1993) 4; 27-34 describe the use of adenovirus based vectors in the delivery of the cystic fibrosis transmembrane conductance product (CFTR) into cells.

It has also been found that the protein of Seq. ID No. 2 is distributed in the cell in both the cytoplasm and the nuclei. In order for a protein to be transported from the cytoplasm to the nuclei, it is usual for processing of the protein to occur, for example by phosphorylation or proteolytic clipping. Compounds which inhibit phosphorylation are known in the art. Compounds which inhibit proteolytic clipping may include peptides of the invention which act as antagonists to inhibit enzymes responsible for clipping. Thus, in a further aspect, the present invention provides a compound which inhibits phosphorylation or proteolytic clipping for use in a method of inhibiting transport of the protein of Seq. ID No. 2 from the cytoplasm to the nucleus of a cell.

The following examples describe the isolation and characterization of the novel protein and DNA of the invention from a murine source. However, other sources, e.g. human or other mammalian are within the scope of the present invention and the human or other mammalian homologues of the protein may be isolated in an analogous manner.

EXAMPLESSection AAffinity purification of DRTF1/E2F from F9 EC cells

5

Polypeptides in affinity purified DRTF1/E2F (approximately 5 μ g from about 5×10^{10} F9 EC cells) were separated by SDS gel electrophoresis and stained with coomassie blue as shown in Figure 1 (track 2); track 1 shows the molecular weight standards.

10 DNA binding polypeptides in affinity purified DRTF1/E2F were assayed by cross-linking to the adenovirus E2A promoter distal E2A site (binding site details indicated in the figure) either in the absence (track 4) or presence of competing wild-type (track 5) or mutant (track 6) E2F binding sites; molecular weight 15 standards are shown in track 3. The p46 polypeptide is indicated; the upper bracket shows another group of polypeptides which also specifically bind to the E2F site of approximate molecular weight 55,000.

Methods: DRTF1/E2F was affinity purified from whole cell 20 extracts prepared from F9 EC cells as described by Shivji and La Thangue (1991) Mol. Cell. Biol. 11, 1686-1695 using affinity matrices containing the wildtype E2F site taken from the adenovirus E2A promoter (-71 to -50) with inclusion of an additional step that involved application of the binding activity 25 to a matrix containing a mutant E2F binding site (mutated in nucleotides -62 to -60). On average about 5.0 μ g of protein were purified from a whole cell extract prepared from about 10^{10} F9 EC cells. UV-crosslinking of DRTF1/E2F to the E2F site was performed using about 50ng of affinity purified protein. Competition was 30 performed with about 100-fold molar excess of either the wildtype (-71 to -50) or mutant (mutated in nucleotides -62 to -60) oligonucleotides.

Nucleotide sequence of a cDNA encoding murine DP-1

Affinity purified DRTF1 was precipitated with TCA, electrophoresed through an SDS polyacrylamide gel and p46 was electroeluted; the purity of p46 was confirmed by electrophoresis and silver staining a small sample of the eluted material. p46 was digested with a lysylendopeptidase, and peptides purified by high performance liquid chromatography using Aquapore AX-300 and RP-300 in series in 0.1% TFA with a 1%/min acetonitrile gradient. Peak fractions were sequenced by automated Edman degradation in a 477A/120A gas-liquid pulse sequencer (Applied Biosystems Inc.).

A set of degenerate oligonucleotide primers were synthesised on the basis of the amino acid sequence in peptides 6 (75 to 91) and 5 (235 to 249) as follows: the C-terminal regions of peptide 6 (P N T H F V), 5' CGCGGATCCCC (ACGT) AA (CT) AC (ACGT) CA (CT) TT (CT) GT 3' and peptide 5 (A Q E S Q N), antisense strand 5' CGCGGATCCA (AG) (AG) TT (CT) TG (ACGT) (CG) (AT) - (CT) TC (CT) TG (ACGT) GC 3'; both oligonucleotides included a linker sequence at the 5' end. Peptide 5 antisense oligonucleotide was used to synthesise cDNA from F9 EC cell RNA which was then used in a PCR with both peptide 6 and 5 primers. Products were subcloned, sequenced and cDNAs derived from DP-1 RNA identified by the presence of peptides 21 and 3, two further peptides obtained by microsequencing peptides derived from p46, which are located between peptides 6 and 5. This cDNA fragment was used to screen several murine cDNA libraries. DP-1 cDNA clones were frequently rearranged and the final nucleotide sequence shown in the figure was obtained from cDNAs isolated from an F9 EC cell library.

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DP-1 is a DNA binding polypeptide in DRTF1/E2F and associates with pRb in vivo.

Peptide 15 (representing DP-1 amino acid residue 235 to 249) and peptide A (representing DP-1 amino acid residue 3 to 15) were coupled to KLH and used to immunise rabbits. The generation of antibodies and immunoblotting were performed by standard procedures. In gel retardation assays about 5.0ng of affinity purified DRTF1/E2F or about 5.0µg of F9 EC crude cell extract were assayed with either the wildtype E2A promoter (-96 to 68) or an oligonucleotide containing E2A promoter sequences -71 to -50 in the presence of about 100-fold molar excess of -62/-60 (E2A sequences -71 to -50 mutated in positions -62, -61 and -60 (ref.23). Anti-peptide A or preimmune sera were added during the preincubation period. Immunoprecipitation of pRb from JM whole cell extracts was performed by standard procedures. The presence of pRb in IF8 immunoprecipitates was confirmed by immunoblotting. The results are shown in Figure 2 as follows:

a) DP-1 is present in affinity pure DRTF1/E2F: immunoblot with affinity purified DRTF1/E2F and anti-peptide 15 (amino acid residues 235 to 249). Reactivity with p46 and p55 was only present in the immune (I, track 2) but not preimmune (PI, track 3) serum. This was specific because it was competitively inhibited by peptide 15 but not an unrelated peptide, peptide 1 (compare tracks 6 and 5). p46 is likely to be a derivative of p55 (indicated in track 3) because anti-peptide A (see below, derived from the N-terminal region of DP-1) only detected p55 (data not shown). Molecular weight standards are shown in track 1, and the * indicates a non-specific reaction.

b) DP-1 is in the affinity pure DRTF1/E2F DNA binding complex: gel retardation was performed with affinity purified DRTF1/E2F and the adenovirus E2A promoter (-96 to +68) in the presence of immune (tracks 3 to 6) or preimmune (track 2) anti-peptide A together with either unrelated peptide 1 (tracks 4 and 6) or peptide 15 (track 5). The specificity of the binding reaction was confirmed by including 100-fold molar excess of the wild-type E2F

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binding site (E2A promoter -71 to -50) in the reaction (track 6); all other reactions contained 100-fold excess of the mutant site.

c) DP-1 is in the DRTF1/E2F DNA binding complex in F9 EC cell extracts: gel retardation was performed in F9 EC whole cell extracts (in which DRTF1/E2F resolves as complexes a,b and c) with an E2F binding site (E2A promoter sequences -71 to -50) in the presence of either preimmune (track 2) or immune (track 3) anti-peptide A serum. The super shift was prevented by including peptide A in the binding reaction (compare duplicate tracks 7 and 8 to 9 and 10).

d) DP-1 associates with pRb in vivo: an immunoprecipitation was performed from JM cell extracts with either the anti-pRb monoclonal antibody IF8 or a control monoclonal antibody A7. The immunoprecipitates were treated with 1.0% deoxycholate (DOC) and the detergent-released material assayed for DRTF1/E2F activity in a gel retardation as described above. DRTF1/E2F binding activity was only detected in the anti-pRb immunoprecipitates (compare tracks 2 to 3); the JM cell extract depleted with anti-pRb had reduced levels of the pRb complex (compare tracks 4 to 5, complex indicated by a). The detergent-released DRTF1/E2F was further assayed for reactivity with anti-peptide A; the immune but not the preimmune serum produced a super shift (compare tracks 6 to 7; indicated by *).

DP-1 is a sequence specific DNA binding protein

Regions of the DP-1 cDNA were amplified in a PCR and subcloned into pGEX-2T (14). GST-DP-1⁸⁴⁻²⁰⁴ and GST-cdk2 were 5 affinity purified on glutathione-Sepharose as previously described by Bandara *et al* (1991) Nature, 352,249-251 Gel retardation was performed as described above with either the E2A promoter or an oligonucleotide containing E2A sequences from -82 to -50; the mutant binding site had nucleotides -62,-61 and -60 10 altered. UV-crosslinking was performed with about 5.0µg of GST fusion protein.

a) The GST fusion protein described in the cartoon, GST-DP-1⁸⁴⁻²⁰⁴, which contains DP-1 protein sequence from amino acid residue 84 15 to 204, was expressed and affinity purified to homogeneity. Track 1 shows the affinity purified DP-1⁸⁴⁻²⁰⁴ and track 2, the standard molecular weight markers.

b) DP-1 binds to the E2F binding site: affinity purified GST-DP- 20 1⁸⁴⁻²⁰⁴ (about 1.0µg) was incubated with either the E2A promoter (track 2), or oligonucleotides (WT and MT binding site details at the bottom of the figure) containing E2A promoter sequence -82 to -50 (track 5) or the same containing a mutant E2F site (track 8). A control GST fusion protein, GST-cdk2 (about 1.0µg), had no 25 binding activity (tracks 3,6 and 9); tracks 1,4 and 7 contain no protein.

c) Crosslinking DP-1 to DNA: affinity purified GST-DP-1⁸⁴⁻²⁰⁴ (track 4) or GST-cdk2 (track 3) was incubated with E2A promoter 30 sequences -82 to -50 (indicated at bottom of tracks). After crosslinking, polypeptides were resolved by SDS gel electrophoresis; crosslinked GST-DP-1⁸⁴⁻²⁰⁴ is indicated by the bracket. No fusion protein was added in track 2 and the molecular weight standards are indicated in track 1.

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d) Binding properties of DP-1 in affinity purified DRTF1/E2F: binding of purified GST-DP-1⁸⁴⁻²⁰⁴ or the control fusion protein GST-cdk2 to the E2A promoter was assayed either alone (tracks 5 and 6; about 1.0µg) or in the presence of affinity pure DRTF1/E2F

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(tracks 3 and 4; about 5.0ng); the binding activity of the same amount of affinity pure DRTF1/E2F is assayed alone in track 2; the E2A promoter is shown in track 1. Note that the binding characteristics of GST-DP-1⁸⁴⁻²⁰⁴ and DRTF1/E2F are different when 5 assayed together (compare tracks 2,3 and 5).

Comparison of DP-1 and E2F-1

This is shown in Figure 4 as follows:

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a) Line diagram of DP-1 and E2F-1 showing the location of the DNA binding domains (amino acid residues 84 to 204 in DP-1, and 89 to 191 in E2F-1; see ref.24) and a region of significant similarity (amino acid residues 163 to 236 in DAP-1, and 162 to 226 in E2F-15 1). Note that the region of similarity includes sequences outside the DNA binding domain.

b) Alignment of amino acid sequence: comparison of DP-1 (top) and E2F-1 (bottom) sequence in regions of similarity. Bars indicate 20 identical (bold) or similar (light) amino acid residues.

c) The DP-1 and E2F-1 regions of similarity form an amphipathic α helix. Presentation of DP-1 (right; amino acids 167 to 183) and E2F-1 (left; amino acids 166 to 182) regions of similarity as a 25 helical wheel.

DP-1 activates E2F site-dependent transcription in vivo.

Either 2.0 or 6.0 μ g of pG4, or 2.8 or 8.8 μ g of pG-B9 (DP-1 30 expression vector) were co-transfected with either p3xWT (5.0 μ g), p3xMT (5.0 μ g) or pCMVcat (0.5 μ g) into SAOS-2 cells as indicated in Figure 5 (constructs indicated in a).

p3xWT and p3xMT reporter constructs contain either three wildtype or three mutant E2F binding sites, taken from the adenovirus E2A 35 promoter (-71 to -50, mutated at nucleotides -62, -61 and -60), positioned upstream of the minimal herpes simplex virus thymidine kinase promoter, and have been described previously. pCMVcat contains the enhancer and promoter region (-301 to +72) taken from the immediate early gene of human cytomegalovirus. pG-B9

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contains DP-1 protein sequence from amino acid residue 63 to 429, and was prepared by replacing the Gal4 DNA binding domain in pG4mpolyII with the DP-1 cDNA B9. Transfection into SAOS-2 cells was performed, and CAT activity was determined and TLC plates 5 quantitated by phosphorimager.

Isolation of a cDNA encoding DP-1

DRTF1 was purified from F9 EC whole cell extracts using a high stringency procedure that involved sequential applications 10 to a DNA binding site affinity matrix containing either a wild-type or mutant binding site, and assaying the DNA binding activity by gel retardation (26). The DNA binding site was taken from the adenovirus E2A promoter (-71 to -51), a region that contains a high affinity E2F binding site (23). This procedure 15 routinely purified a group of polypeptides (Figure 1, track 2) that were capable of efficiently activating transcription in vitro in a binding site-dependent fashion. Several polypeptides in the affinity purified material specifically bound to the wildtype but not to the mutant E2F site (Figure 1, compare tracks 20 5 to 6). The most abundant polypeptide that specifically bound had an apparent molecular weight of about 46kD (Figure 1, track 2, indicated as p46). Subsequently, p46 was excised, digested with a lysylendopeptidase, and the resulting peptides purified and sequenced; the amino acid sequence for ten peptides was 25 obtained. We predicted the DNA sequences that encode two of the peptides and then used these as oligonucleotide primers to amplify a cDNA fragment derived from F9 EC RNA. Several murine cDNA libraries were screened with the cloned cDNA fragment, and a clone representing the complete coding sequence of p46 (from 30 now on referred to as DP-1) finally isolated from an F9 EC cDNA library.

Molecular properties of DP-1

The complete DP-1 coding sequence was determined from a 35 2.4kb cDNA fragment. This cDNA contained an open reading frame encoding 429 in frame amino acid residues that included eight of the peptides obtained from sequencing p46. The cDNA sequence probably includes the initiating methionine because an inframe termination codon exists immediately upstream of it and,

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moreover, the nucleotides flanking and including this methionine would be an efficient translation initiation signal. The longest cDNA clone isolated so far extends 55 nucleotides 5' from this predicted initiating methionine. However, it does not extend to the transcription initiation site because primer extension analysis with F9 EC cell RNA has indicated that the initiation site is 250 nucleotides upstream. The cDNA also contains about 1.1kb of 3'untranslated sequence, part of which is presented.

By northern analysis of poly adenylated F9 EC RNA the size of the DP-1 transcript was estimated to be about 2.6kb. It was observed that DP-1 RNA is constitutively expressed in many different cell types and when assessed by *in situ* hybridization in a wide range of tissues during murine embryogenesis. During F9 EC cell differentiation, it was found that DP-1 RNA is marginally down-regulated as F9 EC stem cells differentiate. Southern analysis indicated that DP-1 is encoded by a single gene.

Homology searches of the currently available protein data bases (Leeds and Swiss) failed to detect any significant similarity between DP-1 and any other protein. However, we noticed that a small region within the DNA binding domain of DP-1 has significant similarity to an analogous region in the DNA binding domain of E2F-1 (see later), a recently characterised protein that also binds to the E2F site.

Characterisation of DP-1

To confirm that DP-1 is a component of DRTF1/E2F and to determine whether it is present in the DRTF1/E2F DNA binding complex, a number of anti-peptide antisera were raised against different regions of the DP-1 protein (Figure 2). Immunoblotting affinity purified DRTF1/E2F with anti-peptide 15 revealed two polypeptides with apparent molecular weight 46kD and 55kD (Figure 2a, track 2). Both polypeptides were specifically recognised by the antiserum because the reaction was competitively inhibited by peptide 15 but not by an unrelated peptide, peptide 1 (Figure 2a, compare tracks 5 and 6).

It was confirmed that DP-1 was part of the DRTF1/E2F DNA binding complex by determining the effect that another anti-peptide serum (anti-peptide A) had in gel retardation assays;

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anti-peptide 15 could not be used for these assays because it only reacts with the denatured protein. When anti-peptide A was incubated with affinity purified DRTF1/E2F a super-shift was apparent with the immune but not preimmune serum (Figure 2b, compare tracks 2 and 3). This super-shift was specific because it was competitively inhibited by including peptide A but not the unrelated peptide 1 (Figure 2b, compare tracks 4 to 5). Since all the binding activity resolved in these gel retardation conditions was specific for the E2F binding site (Figure 2b, compare track 10 4 to 6), these results indicate that DP-1 is part of the DRTF1/E2F DNA binding activity.

The anti-peptide antibodies also caused a super-shift in whole cell extracts. In F9 EC cell extracts DRTF1 resolves as a series of DNA-protein complexes, referred to as DRTF1a,b, and c. 15 The addition of anti-peptide A, but not the preimmune serum, caused a supershift and a concomitant reduction in DRTF1a,b and c (Figure 2c, compare tracks 2 and 3) which could be competed by peptide A (Figure 2c, compare tracks 7 and 8 to 9 and 10). Similar effects were apparent in a wide variety of whole cell 20 extracts derived from other types of cells, including HeLa. This suggests that DP-1 is a component of the DNA binding activity, DRTF1/E2F, that was initially defined in F9 EC cell extracts and which was also characterised in HeLa cell extracts.

25 DP-1 associates with the retinoblastoma gene product in vivo

DRTF1/E2F binds to pRb both in vitro and in vivo. To determine if DP-1 associates with pRb in vivo an immunoprecipitation experiment was performed with the anti-pRb monoclonal antibody IF8 from whole cell extracts prepared from 30 the human leukaemic cell line JM, which contains high levels of the DRTF1/E2F-pRb complex. After immunoprecipitation, DRTF1/E2F DNA binding activity was released from pRb by treating the immune complex with mild detergent. In these conditions, DRTF1/E2F DNA binding activity was released only from the pRb 35 immunoprecipitate, and not the control antibody immunoprecipitate (Figure 2d, compare tracks 2 and 3). The presence of DP-1 in pRb-associated DRTF1/E2F was confirmed by treating the detergent release with anti-peptide A which caused DRTF1/E2F to supershift

(Figure 2d, compare tracks 6 and 8, indicated by *). This demonstrates that DP-1 associates with pRb in vivo.

DP-1 specifically binds to an E2F site and has a novel DNA
5 binding domain

In order to determine whether DP-1 could bind to the E2F site in a sequence-specific fashion, regions of DP-1 coding sequence were expressed as glutathione-S-transferase (GST) fusion proteins, affinity purified, and tested for DNA binding activity. 10 The smallest region so far defined that retains DNA binding activity contains DP-1 protein sequence from amino acid residue 84 to 204 (Figure 3a; GST-DP-1⁸⁴⁻²⁰⁴); this bound to the adenovirus E2A promoter whereas an unrelated GST fusion (GST-cdk2) failed to do so (Figure 3b; compare tracks 2 and 3). Furthermore, the DNA 15 binding activity was specific for the E2F site because GST-DP-1⁸⁴⁻²⁰⁴ bound more efficiently to the wildtype than to the mutant E2F site (Figure 3b, compare tracks 5 to 8). Thus DP-1 has similar DNA binding specificity as DRTF1/E2F in whole cell extracts and p46 in affinity pure DRTF1/E2F; the control GST fusion protein 20 lacked any DNA binding activity (Figure 3b, compare tracks 6 to 9). An anti-GST serum altered the mobility of the DP-1/DNA complex, confirming the presence of GST-DP-1⁸⁴⁻²⁰⁴ in the DNA binding complex. Furthermore, GST-DP-1⁸⁴⁻²⁰⁴ shared another property with p46 in that it could be specifically cross-linked 25 to the E2F site (Figure 3c, compare tracks 3 to 4). DP-1 therefore binds to the E2F site in a sequence-specific fashion and is thus likely to be a polypeptide in DRTF1/E2F that contributes to the DNA binding specificity.

Attempts to define the DNA binding domain more precisely 30 have proved unsuccessful. Thus, this region of DAP-1 is likely to be close to the minimal amount of protein that allows sequence specific recognition and therefore, in all probability, defines the DNA binding domain. Overall, it is unrelated to any other type of DNA binding structure so far identified, and thus 35 represents a new class of DNA binding domain. A small region (DP-1 amino acid residue 160 to 200) that has significant similarity to a region that lies within the DNA binding domain of E2F-1 (Figure 4a), where 42% of the amino acid residues are identical and 70% similar (Figure 5b) was noted. Secondary structure

predictions suggests that these regions include two α helices, one of which is amphipathic (represented as a helical wheel in Figure 4c). Since DP-1 and E2F-1 bind to the same DNA sequence, this region of similarity appears to be involved in recognising the DNA sequence that constitutes an E2F binding site.

The potential similarity of two other DNA binding proteins that regulate transcription during the yeast cell cycle supports this finding. Thus, the budding and fission yeast cell cycle-regulating proteins, encoded by SWI4 and cdc10, bind to a DNA sequence that resembles the E2F site, and contain a region within their DNA binding domains that has features in common with the DP-1/E2F-1 α helical region discussed above (Figure 4c). Based on the above, this SWI4/cdc10 protein domain may also be involved in DNA sequence recognition.

Another region of similarity between DP-1 and E2F-1 is apparent outside the DNA binding domain (DP-1 amino acid residue 210 to 240, with 41% identical amino acid residues; Figure 4b) which, like the earlier region, may form an amphipathic α helix. This region may contribute to DNA binding activity.

DP-1⁸⁴⁻²⁰⁴ may also contain a protein dimerisation interface because the addition of GST-DP-1⁸⁴⁻²⁰⁴ to affinity purified DRTF1/E2F resulted in a slower migrating protein-DNA complex relative to either GST-DP-1⁸⁴⁻²⁰⁴ or affinity purified DRTF1 alone, whereas a control GST-fusion protein had little effect (Figure 3d). The slower migrating complex is likely to result from the interaction of GST-DP-1⁸⁴⁻²⁰⁴ with another protein in affinity purified DRTF1/E2F through a dimerisation domain contained within DP-1⁸⁴⁻²⁰⁴. The similarity between DP-1 and E2F-1 protein sequences within this region suggests that E2F-1 is a strong candidate for such a dimerisation partner.

DP-1 activates transcription in vivo

The E2A promoter distal E2F site (-71 to -50) functions as an activating sequence in a variety of cell types, such as SAOS-2 and F9 EC cells, when positioned upstream of the minimal herpes simplex virus thymidine kinase promoter. To determine if DP-1 can trans activate transcription through the E2F site, the effect of expressing the DP-1 coding sequence (amino acid residue 63 to 429) on the transcriptional activity of p3xWT and p3xMT, reporter

constructs that are driven by either three wildtype or three mutant E2F binding sites respectively (Figure 5a) was assessed. In SAOS-2 cells, co-transfection of pG-B9 with p3xWT stimulated the transcriptional activity of p3xWT, whereas there was no effect of the plasmid pG4 (Figure 5b). Transcriptional activation by pG-B9 was dependent on wildtype E2F sites because the activity of either p3xMT or pCMVcat was not significantly affected in the same transfection conditions (Figure 5b and c). This shows that DP-1 specifically activates transcription through a wildtype E2F site.

Thus, DP-1 is a sequence specific DNA binding protein that is present in DRTF1/E2F complexes assayed in extracts prepared from a variety of cell types and tissues.

Both pRb and p107 bind to DRTF1/E2F in a cell cycle-dependent fashion, an interaction that causes a reduction in the transcriptional activity of DRTF1/E2F. These complexes, together with the free transcriptionally active form of DRTF1/E2F, can be resolved in extracts from asynchronous cultures of tissue culture cells.

A striking feature of the DP-1 protein is its similar organisation to E2F-1, a recently described protein which also has properties similar to DRTF1/E2F. Thus, their DNA binding domains and regions of similarity are located in very similar positions (Figure 4a) although the rest of the proteins are very different. It appears that DP-1 and E2F-1 exist together in an E2F complex because antibodies against E2F-1 supershift complexes which also contain DP-1. This is reminiscent of the situation that exists in other transcription factor activities, for example AP-1, where very different proteins interact through related domains. It is, however, believed that there is more than one partner for DP-1 because although DP-1 is present in all DRTF1/E2F complexes that form on the E2F binding site, antibody super-shift experiments indicate that E2F-1 is not; perhaps other E2F-1-like polypeptides exist together with DP-1 in these complexes.

SECTION B.

Several lines of evidence suggest that the cellular transcription factor DRTF1/E2F plays an important role in regulating the cell cycle of mammalian cells. For example, DRTF1/E2F DNA binding activity is periodically induced during cell cycle progression, peaking during S phase (Mudryj et al., 1992; Shirodkar et al., 1992), and negatively regulated during differentiation (La Thangue & Rigby, 1987). This binding activity correlates with the transcriptional activity of certain genes that are necessary for cellular proliferation, such as DHFR, DNA polymerase α and p34^{cdc2}, which contain DRTF1/E2F binding sites in their promoters (Blake & Azizkhan, 1989; Means et al., 1992; Dalton, 1992). Furthermore, the retinoblastoma tumour suppressor gene product, which negatively regulates cell cycle progression from G1 into S phase and is frequently mutated in tumour cells, binds to DRTF1/E2F (Bandara and La Thangue, 1991; Chellapan et al., 1991). The functional consequence of this interaction is that pRb prevents DRTF1/E2F from activating transcription (Zamanian and La Thangue, 1992). Several other molecules that are implicated in cell cycle control, such as Rb-related p107, cyclins A and E, and p33^{cdk2} also associate with DRTF1/E2F during cell cycle progression (Bandara et al., 1991, 1992; Mudryj et al., 1991; Devoto et al., 1992; Lees et al., 1992). Taken together, these observations suggest that DRTF1/E2F integrates cell cycle events with the transcription apparatus, ensuring that the cell makes the appropriate changes in gene expression at the correct time during cell cycle progression.

Further evidence for the importance of DRTF1/E2F has come from studies on the mechanism of action of viral oncoproteins. Thus, certain oncoproteins, such as adenovirus E1a, SV40 large T antigen and human papilloma virus E7 regulate the activity of DRTF1/E2F by sequestering pRb and the other associated proteins, converting it from a transcriptionally inactive to an active form (Zamanian and La Thangue, 1992; Hiebert et al., 1992; Zamanian and La Thangue, 1993). Because this effect requires regions in these viral oncoproteins previously shown to be necessary for cellular immortalization and transformation (Bandara and La

Thangue, 1991; Zamanian and La Thangue, 1992), it is likely that DRTF1/E2F plays an important role in these processes.

Although progress has been made in identifying the cellular proteins that interact with DRTF1/E2F, relatively little was, until recently, known about its molecular details. Two distinct polypeptides which are both DNA binding components of DRTF1/E2F have now been molecularly characterised. The first, referred to as E2F-1, was isolated through its ability to directly bind to pRB, which it does through a C-terminal region (Helin et al., 1992; Kaelin et al., 1992). In contrast, DP-1 was defined as a component of DRTF1/E2F DNA binding activity after biochemically purifying DRTF1 from F9 embryonal carcinoma (EC) stem cells, a cell system in which DRTF1/E2F is down-regulated during the differentiation process (La Thangue and Rigby, 1987; La Thangue et al., 1990). cDNAs that encode DP-1 were isolated after obtaining amino acid sequence from affinity purified DP-1 (Girling et al., 1993).

Both E2F-1 and DP-1 contain a region that allows each polypeptide to bind in a sequence-specific fashion as a homodimer to the E2F motif (Helin et al., 1992; Kaelin et al., 1992; Girling et al., 1993). Although the DNA binding domains are not closely related to any previously defined DNA binding structure they are, nevertheless, distantly related to the DNA binding domains in some yeast cell cycle-regulating transcription factors (La Thangue and Taylor, 1993). The functional relationship between DP-1 and E2F-1 has, however, remained unclear. In this study, we show that DP-1 and E2F-1 exist as a complex *in vivo* which recognises the E2F binding site. Moreover, *in vitro* assays demonstrate that DP-1 and E2F-1 bind efficiently and preferentially as a complex to the E2F site, an interaction which requires the region of similarity between the two proteins. Furthermore, reconstructing DRTF1/E2F in *Drosophila* and yeast cells suggests that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation. These data indicate that DP-1 and E2F-1 can functionally interact and that such an interaction is likely to be physiologically relevant in mammalian cells.

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DP-1 and E2F-1 exist as a complex in HeLa cells.

DP-1 is a component of DRTF1/E2F which is present in murine developmentally regulated and cell cycle regulated DRTF1/E2F complexes and thus is likely to be a general component of DRTF1/E2F DNA binding activities. Furthermore, DP-1 is the product of a conserved gene since it has been observed by the present inventor that a closely related protein is expressed in amphibians and *Drosophila*. DP-1 thus appears to be a frequent and evolutionarily conserved DNA binding component of DRTF1/E2F. E2F-1, which was isolated through its ability to bind directly to pRb, also interacts in a sequence-specific fashion with the E2F site (Helin et al., 1992; Kaelin et al., 1992). Both proteins contain a small region of similarity that overlaps domains previously shown to be necessary for sequence-specific DNA binding activity (Girling et al., 1992).

It was assessed whether DP-1 and E2F-1 exist as a complex in HeLa cell extracts using antibodies that specifically recognise each protein. Initially, we determined by gel retardation whether DP-1 is a component of HeLa cell DRTF1/E2F. Thus, as in F9 embryonal carcinoma (EC) cell extracts, anti-DP-1 peptide antiserum disrupted HeLa cell DRTF1/E2F in a specific fashion since its effects were competed by including in the binding reaction the homologous, but not an unrelated, peptide (Figure 6a, compare tracks 2 through 5 with 6 through 9). Anti-DP-1 antiserum was used to immunoprecipitate DRTF1/E2F from HeLa cell extracts, the immunoprecipitate subsequently being released and then immunoblotted with an anti-E2F-1 monoclonal antibody. The DRTF1/E2F DNA binding activity immunoprecipitated by anti-DP-1 (Figure 6b, compare tracks 4 and 7) contained the E2F-1 protein because immunoblotting the immunoprecipitates with an anti-E2F-1 monoclonal antibody revealed a polypeptide with the molecular weight expected for E2F-1 (Figure 6c, track 4, indicated by arrow). The presence of E2F-1 was dependent upon the anti-DP-1 activity since it was not present when the immunoprecipitation was performed in the presence of the homologous peptide (Figure 6c, compare tracks 3 and 4). Thus, DP-1 and E2F-1 exist as a complex in HeLa cell extracts.

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DP-1 and E2F-1 interact *in vitro* in a DNA binding heterodimer. Both DP-1 and E2F-1 contain sequence-specific DNA binding domains, located in similar positions of each protein (between amino acid residue 84 and 204 in DP-1, and 89 to 191 in E2F-1; Girling et al., 1993), which contain a region of similarity that extends outside of the DNA binding domain, to amino acid residue 249 in DP-1. In agreement with previous studies (Helin et al., 1992; Kaelin et al., 1992; Girling et al., 1993) both DP-1 and E2F-1 alone were able to bind to the E2F site, either in the context of the adenovirus E2A promoter (Figure 7a, tracks 2 and 3) or as a single E2F site (which was apparent on increased exposure of Figure 7a, track 6; data not shown). The DNA binding activity of DP-1 was somewhat less than that of E2F-1, the reasons for which are currently unclear. However, when both proteins were present in the same binding reaction, increased E2F site DNA binding activity was apparent (Figure 7a, compare tracks 2 and 3 with 4, and 6 and 7 with 8). The DNA binding activity was much greater than that expected from an additive effect of the two DNA binding activities, indicating that together DP-1 and E2F-1 recognise the E2F site synergistically.

The presence of both DP-1 and E2F-1 in the DNA binding complex was confirmed using antisera specific for either protein. An anti-E2F-1 peptide antiserum supershifted the DNA binding complex (Figure 7a, compare track 8 with 10), whereas the anti-DP-1 peptide antiserum inhibited the DNA binding activity (Figure 7a, compare track 11 and 12). However, the effect of the anti-DP-1 antiserum was less dramatic, the reasons for which are unclear, but may be related to the availability of the epitope which, for this antibody, is located close to the DNA binding domain of DP-1 (Girling et al., 1993).

This assay was used to determine the regions in DP-1 which are necessary to produce a DNA binding complex with E2F-1. Thus, various derivatives of DP-1 were expressed as GST fusion proteins, cleaved with thrombin, and then assessed for any interaction with E2F-1. Since these derivatives of DP-1 were truncated versions of the wild-type protein, any of them which was able to interact with E2F-1 to produce functional DNA binding

activity should result in a smaller and hence faster migrating DNA binding complex. Moreover, if only one faster migrating complex were apparent, a heterodimer of the two proteins would be the most likely explanation. Indeed, when either DP-1⁸⁴⁻²⁴⁹ or DP-1⁸⁴⁻²⁰⁴ were mixed with E2F-1 (GST-E2F-1⁸⁹⁻⁴³⁷), a faster migrating DNA binding complex was formed relative to E2F-1 alone (Figure 7b, compare track 1 with 2 and 3) or E2F-1/DP-1 (Figure 7a) indicating that these two derivatives of DP-1 were able to interact with E2F-1 and that they were likely to form a heterodimer. Again, the DNA binding activity of the E2F-1/DP-1⁸⁴⁻²⁴⁹ complex was greater than that for E2F-1 alone (Figure 7b, compare track 1 with 2 and 3) or DP-1⁸⁴⁻²⁴⁹ which had low DNA binding activity in the conditions employed in this assay (data not shown) but nevertheless can specifically recognise the E2F site (Girling et al., 1993). The DNA binding activity of the E2F-1/DP-1⁸⁴⁻²⁰⁴ reaction was less than E2F-1/DP-1⁸⁴⁻²⁴⁹ indicating that the region of DP-1 between amino acid residue 204 and 249, which shows significant similarity to E2F-1 (Girling et al., 1993), also influences DNA binding activity. The synergistic DNA binding effects of DP-1⁸⁴⁻²⁴⁹ and DP-1⁸⁴⁻²⁰⁴ were also apparent when the uncleaved GST fusion proteins were mixed with E2F-1 although, because of their increased size, a faster migrating DNA binding complex did not occur (Figure 7b, compare track 1 with 4 and 5). Further deletion of this region, either from the N- (DP-1¹⁴⁶⁻²⁴⁹) or C- (DP-1⁸⁴⁻¹⁶⁶) terminus yielded derivatives of DP-1 that failed to form a DNA binding complex with E2F-1 either as GST fusion proteins (Figure 7b, compare track 1 with 6 and 7) or after cleavage (data not shown), indicating that DP-1⁸⁴⁻²⁰⁴ is the minimal region so far defined which is capable of producing a DNA binding complex with E2F-1.

Analysis of the DNA binding specificity of the E2F-1/DP-1⁸⁴⁻²⁴⁹ complex with a panel of binding sites derived from the adenovirus E2A promoter distal E2F site (La Thangue et al., 1990; Shivji and La Thangue, 1991) indicated that it was very similar to that for E2F-1 alone (Figure 7c, compare tracks 3 through 6 with 8 through 11) and, furthermore, the DRTF1/E2F site DNA binding activity defined in F9 EC cell extracts (Figure 7c, compare tracks 13 through 16).

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To characterise further the interaction between DP-1 and E2F-1 we employed an assay in which *in vitro* transcribed and translated E2F-1 polypeptide could bind to DP-1 GST fusion proteins. The ability of E2F-1 to interact with DP-1 was assessed after collecting the GST-fusion protein with glutathione-agarose beads and subsequently releasing the bound E2F-1 polypeptide. Both DP-1⁸⁴⁻²⁴⁹ and DP-1⁸⁴⁻²⁰⁴ could interact with E2F-1 since the amount of E2F-1 bound to GST-DP-1⁸⁴⁻²⁴⁹ and GST-DP-1⁸⁴⁻²⁰⁴ was significantly greater than that bound by the GST beads alone (Figure 7d, compare track 2 to 3 and 6), consistent with their ability to form a DNA binding heteromer (Figure 7b). DP-1¹⁴⁶⁻²⁴⁹ also bound to E2F-1 whereas DP-1⁸⁴⁻¹⁶⁶ failed to do so (Figure 7d, compare tracks 2 to 4 and 5). DP-1¹⁴⁶⁻²⁴⁹ therefore contains a domain, which based on the earlier results is likely to be a dimerization domain, that allows it to interact with E2F-1 but lacks sufficient amino acid sequence for the heteromer to bind to DNA. The additional information in DP-1⁸⁴⁻²⁴⁹ is necessary for the complex to bind to DNA. These data therefore suggest that the region of DP-1 which is similar to E2F-1 (amino acid 163 to 236) contains a dimerization domain, and that additional N-terminal sequence is necessary for DNA binding activity. A summary of these data is presented in Figure 7e.

DP-1 and E2F-1 interact in yeast cells.

To determine if DP-1 and E2F-1 interact directly *in vivo* we adapted a previously described assay system in yeast cells (Fields and Song, 1989) which utilised expression vectors that synthesise two hybrid proteins, one derived from DP-1 and the other from E2F-1. In the first, the DP-1 coding sequence was fused to the DNA binding domain of the bacterial LexA protein, to make pLEX.DP-1 and in the second, pGAD.E2F-1, the E2F-1 coding sequence was fused with the acidic transcriptional activation domain (AAD) of the yeast Gal4 protein. pLEX.DP-1 failed to activate a reporter construct driven by a LexA binding site, whereas a hybrid protein that contained the trans activation domain taken from the p53 protein could (Figure 8). However, when pLEX.DP-1 and pGAD.E2F-1 were expressed together, the transcriptional activity of the LexA reporter construct was increased considerably (about 75-fold) relative to its activity

when either pLEX.DP-1 or pGAD.E2F-1 were expressed alone (Figure 8). This result, combined with the earlier studies presented in this paper, strongly suggest that DP-1 and E2F-1 interact directly *in vivo*.

DP-1 regulates E2F site-dependent transcription *in vivo*.

Increasing the levels of the DP-1 protein in a variety of mammalian cells (for example, F9 EC, SAOS-2 and 3T3) and growth conditions failed to significantly stimulate the transcriptional activity of an E2F site-dependent reporter (data not shown). In order to assess if DP-1 and E2F-1 functionally interact we therefore had to take an alternative approaches.

(a) *Drosophila* assay.

A first approach involved developing the appropriate assay in *Drosophila* SL2 cells, a cell system which has been used previously to study the activity of mammalian transcription factors (Courey and Tjian, 1988). These cells were particularly appropriate for this analysis because the endogenous E2F site DNA binding activity is very low when assayed by gel retardation (data not shown). In order to assess the functional interaction of DP-1 and E2F-1, we determined the effects of each protein alone and when expressed together on the transcriptional activity of p3xWT, a reporter construct driven by three E2F sites (Figure 9a; Zamanian and La Thangue, 1991). Thus, E2F-1 was able to activate p3xWT in a dose-dependent fashion (Figure 9b and c, compare lanes 1 and 2) whereas DP-1 failed to do so (Figure 9b and c, compare lanes 3 and 4), results which are similar to the behavior of E2F-1 and DP-1 in mammalian cells (Helin et al., 1992; Kaelin et al., 1993; and data not shown). However when DP-1 and E2F-1 were expressed together much greater E2F site-dependent transcriptional activation was apparent relative to either alone (Figure 9b and c, compare lanes 1, 3 and 5). Moreover, this synergistic effect was titratable because increasing the level of DP-1 produced more E2F site-dependent transcription (Figure 9b and c, compare lanes 1, 5 and 6) and specific since co-expression of an unrelated DNA binding, derived from the Gal4 protein, did not produce any significant effects (Figure 9b and c, compare lanes 5 and 6 with 7 and 8). Moreover,

similar experiments performed with p3xMT indicated that this activation was specific for the wild-type E2F site (data not shown). It therefore appears that DP-1 and E2F-1 functionally interact in E2F site-dependent transcription and that this interaction is synergistic.

(b) F9 EC cell assay.

In an alternative approach a "dominant negative" derivative of DP-1 was designed which could cripple the endogenous DRTF1/E2F transcriptional activity. DP-1⁷³⁻³⁴⁰ was prepared which lacks amino acid sequence from both the N and C-terminal region. Thus, when DP-1⁷³⁻³⁴⁰ was expressed in F9 EC cell, which contain high levels of E2F site-dependent transcriptional activity (Zammanian and La Thangue, 1992, a reduction in the activity of p3xWT was apparent (Fig. 10c, compare tracks 1 and 2 to 7 and 8, quantitated at the bottom of the figure); this effect was specific for the wild type E2F site because the activity of either the mutant E2F site reporter p3xMT or pCMVcat was not affected. Based on the earlier results, a potential explanation for the effect of DP-1⁷³⁻³⁴⁰ was that it sequestered E2F-1, to form a heteromer that was not capable of activating transcription, thus limiting the amount of available E2F-1. When E2F-1 was expressed in the presence of DP-1⁷³⁻³⁴⁰, it overcame the "dominant negative" activity and partially restored E2F site-dependent transcriptional activity (Fig. 10, compare tracks 7 and 8 to 9 and 10). The ability of E2F-1 to rescue E2F transcriptional activity is consistent with the idea that DP-1⁷³⁻³⁴⁰ sequesters E2F-1 into an inactive transcription complex and is compatible with the idea that an interaction between DP-1 and E2F-1 occurs in mammalian cells.

DP-1 and E2F-1 activate E2F site-dependent transcription yeast cells.

DP-1 and E2F-1 were then assessed to determine if they can functionally interact in E2F site-dependent transcription in yeast cells. For this, constructs in which the yeast *cycl* promoter was driven by E2F binding sites taken from the adenovirus E2A promoter were used. In p4xWT CYC1, four E2F binding sites drive the *cycl* promoter (Figure 11a), activating

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transcription about 12-fold above the activity of p4xMT CYC1 (data not shown). This transcriptional activity could be stimulated further upon introduction of the E2F-1 expression vector, pGAD.E2F-1. Thus, pGAD.E2F-1 increased the transcriptional activity of p4xWT CYC1 about 10-fold, compared to the small effect that the DP-1 expression vector, pLEX.DP-1, had on the same reporter construct (Figure 11b). However, when E2F-1 and DP-1 were expressed together, the activity of p4xWT CYC1 was even greater, and usually about 50-fold above basal p4xWT CYC1 activity (Figure 5b); the activity of p4xMT CYC1 was not significantly affected by either the E2F-1 or DP-1 expression vector (data not shown). This shows that DP-1 and E2F-1 activate E2F site-dependent transcription more efficiently when present together than either does alone, suggesting again that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation.

DP-1 and E2F-1 interact in mammalian cells.

Previous studies have indicated that DP-1 is a universal component of DRTF1/E2F DNA binding activity in F9 EC cells because all the DNA binding complexes that occur on the E2F site are disrupted by anti-DP-1 antibodies (Girling et al., 1993). The same situation exists in HeLa cell extracts where all the DRTF1/E2F DNA binding complexes are affected by anti-DP-1 antibodies (Figure 6a). Based on these observations, and combined with studies performed in other cell types (Bandara et al., in preparation), we believe that DP-1 is a frequent component of transcription factor DRTF1/E2F.

In the light of these observations, we were interested to determine if DP-1 can interact with the other E2F site DNA binding protein, E2F-1 (Helin et al., 1992; Kaelin et al., 1992) and, furthermore, establish whether such an interaction occurs in physiological conditions. Our results indicate that DP-1 and E2F-1 exist as a complex in HeLa cell extracts, and thus imply that at least a proportion of the total DRTF1/E2F DNA binding activity is likely to be a heteromeric complex involving DP-1 and E2F-1. It is unclear, at the moment, just how much of the DRTF1/E2F DNA binding activity is a complex of DP-1 and E2F-1

because our attempts to use anti-E2F-1 antibodies to affect the DNA binding activity in gel retardation assays have been unsuccessful (data not shown). Also, we cannot rule out that other proteins bind to DP-1, in the place of E2F-1. In fact, this would seem a likely possibility because several polypeptides in affinity purified DRTF1/E2F with distinct molecular weights (from 45 to 55,000) are capable of specifically binding to the E2F site (Shivji and La Thangue, 1991; Girling et al., 1993).

A physical interaction between DP-1 and E2F-1 in vitro and in yeast cells.

We established that DP-1 and E2F-1 can directly interact by studying their DNA binding properties in gel retardation assays. DP-1 and E2F-1 formed a heteromeric DNA binding complex with exactly the same DNA binding specificity as that possessed by DRTF1/E2F in crude cell extracts (La Thangue et al., 1990). Moreover, it was apparent that the DNA binding activity of the heteromer was considerably greater than for E2F-1 or DP-1 alone, suggesting that DP-1 and E2F-1 interact synergistically. A molecular analysis of the region in DP-1 which was necessary to form a DNA binding complex with E2F-1 indicated that the region of similarity between the two proteins, together with an additional N-terminal domain, was required. The region of similarity allowed DP-1 and E2F-1 to bind to each other and thus is likely to constitute a dimerization domain.

We confirmed these observations in yeast cells using an assay which makes use of the modular organisation of transcription factors (Fields and Song, 1989). Thus, DP-1 was fused to the bacterial LexA DNA binding domain and, in a separate molecule, E2F-1 to the acidic transcriptional activation domain of the yeast Gal4 protein. In this assay, a functional activation domain is recruited to the LexA-dependent promoter only if there is a physical interaction between the two hybrid proteins. When the two hybrid proteins were expressed together there was strong activation of the LexA-dependent reporter. Thus, DP-1 and E2F-1 are able to physically interact in yeast cells. Moreover, this result indicates that they are able to do so in the absence of

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DNA binding since the DNA binding specificity was provided by LexA and thus took place independently of the E2F binding site.

Transcriptional synergy by DP-1 and E2F-1 *in vivo*.

We addressed the functional consequences of the interaction between DP-1 and E2F-1 for E2F site-dependent transcription in both *Drosophila* and yeast cells. We took this approach because our attempts to activate transcription by introducing wild-type DP-1 into mammalian cells have met with limited success, the reasons for which are unclear but may be related to the levels of endogenous DP-1 protein.

Both types of assay, whether performed in *Drosophila* or yeast cells, indicated that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcription since when both proteins were expressed together transcriptional activation was more efficient than for either protein alone. A likely explanation for such an effect is that the DNA binding activity of the DP-1/E2F-1 heterodimer is more stable than either homodimer and thus transcriptional activation is more efficient. This idea would be entirely consistent with the *in vitro* DNA binding data presented earlier in this study which suggested that DP-1 and E2F-1 interact synergistically. We cannot, however, rule out other potential influences, such as activation of a cryptic transcriptional activation domain in the DP-1/E2F-1 heterodimer and, in fact, recent experiments have suggested that such a possibility is likely to be correct (Zamanian and La Thangue, unpublished data).

In conclusion, we have demonstrated that DP-1 and E2F-1 interact in transcription factor DRTF1/E2F, to produce a DNA binding complex which is the preferred state over either homodimer. Since E2F-1 can bind to pRb (Helin et al., 1992; Kaelin et al., 1992) in such a complex it is likely that E2F-1 will provide an interface recognised by pRb, thus enabling the transcriptional activity of this particular E2F site DNA binding activity to be regulated by pRb. It is possible that other molecules heterodimerize with DP-1, in the place of (and perhaps related to) E2F-1, providing an interface recognised by other proteins

which are known to interact with E2F/DRTF1, such as p107 (Zamanian and La Thangue, 1993), thus allowing these molecules also to regulate E2F site-dependent transcription. We suggest therefore that distinct heterodimers recognise the E2F site, with DP-1 as a common component, enabling different molecules, such as pRb and p107, to integrate their biological activities with the transcription apparatus and hence to regulate genes driven by E2F/DRTF1.

MATERIALS AND METHODS

Preparation of cell extracts, gel retardation and immunochemical techniques.

Cell extracts were prepared as previously described (La Thangue et al., 1990). Gel retardation in F9 EC and HeLa cell extracts (about 6.0 µg) in the presence of anti-DP-1 was performed as previously described (Girling et al., 1993), and immunoprecipitation with anti-DP-1 from HeLa cell extracts was performed by standard procedures. The immunoprecipitates were treated with 1% DOC and 1.5% NP40 and the detergent released material assayed for DRTF1/E2F by gel retardation and the presence of E2F-1 by immunoblotting with the anti-E2F-1 monoclonal antibody SQ41 (Kaelin et al., 1992). The anti-DP-1 antibodies, anti-peptide A and anti-peptide 18, have been previously described (Girling et al., 1993). Rabbit anti-E2F-1 antiserum (antiserum 134) was raised against a peptide which represents E2F-1 amino acid sequence 315 to 323. The sequences of the binding sites used to assess DNA binding specificity were derived from the adenovirus E2A promoter (-71 to -50) and were as follows: WT; TAGTTTTTCGCGCTTAAATTGA; 62/60, TAGTTTTTCGATATTAAATTGA; 63, TAGTTTTCTCGCTTAAATTGA; 64, TAGTTTTAGCGCTTAAATTGA. In figure 2a (tracks 1 to 4), the adenovirus E2A promoter (-96 to + 68) was used; in all other cases, the distal E2F site in the E2A promoter (sequences -71 to -50) was used. About 100-fold excess of competing binding sites were used in the gel retardation assays.

Fusion proteins and *in vitro* translation.

DP-1 and E2F-1 were expressed as, and released from, GST fusion proteins as previously described (Girling et al., 1993). About 100-fold excess of the competing binding sites were used in gel retardation assays, with the binding site taken from the adenovirus E2A promoter (-71 to -50). The wild-type E2F-1 coding sequence was transcribed and further translated using reticulocyte lysate (Promega) and radiolabelled with ³⁵S methionine. In the dimerization assay (Figure 2d), GST-DP-1 fusion protein was incubated with E2F-1 polypeptide for 30 min at 30°C, collected with glutathione-agarose (Sigma), and washed repeatedly with 0.1% NP40 in PBSA. Bound E2F-1 polypeptide was released by denaturation in SDS sample buffer and resolved in a 10% polyacrylamide gel.

Yeast assays.

pBTM116 contains the complete LexA coding sequence (1-202) under the control of the yeast *ADH1* promoter. pLEX.DP-1 carries the coding sequence for DP-1 (from amino acid 59 to the C-terminus) downstream of the LexA coding sequence in pBTM116. pBTM126 carries the wild-type murine p53 coding sequence (from amino acid 1 to 346) downstream of the LexA DNA binding domain. pGAD.L6 is a derivative of pGAD2F (Chien et al., 1991) containing the Gal4 transcription activating domain (from amino acid residue 768-881) under the control of yeast *ADH1* promoter. pGAD.E2F-1 contains the entire E2F-1 coding sequence (from amino acid 1 to 437) downstream of the Gal4 activation domain. p4xWT CYC1 and p4xMT CYC1 were derived from pLGA178 (Guarente and Mason, 1983). The wild-type E2F site was taken from the -71 to -50 region of the adenovirus E2A promoter and the mutant site was mutated in nucleotides -62 to -60 (La Thangue et al., 1990). For the yeast interaction assay (Figure 3), the indicated expression vectors were transformed into the yeast strain CTY10-5d (*MATa ade2 trp1-901 leu2-3, 112 his3-200 gal4 gal80 URA3::lexAop-lacZ*) which contains an integrated plasmid which carries 2 copies of a 78-bp oligonucleotide, each copy containing two colE1 operators or four binding sites for LexA dimers upstream of the transcription start site of *GAL1-lacZ*. For the yeast E2F site-dependent transcription assay (Figure 5), the yeast strain W3031a (*MATa ade2-100 trp1-1 leu2-3 112 his3-11 ura3*) was used carrying either

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p4xWT CYC1 or p4xMT CYC1 and was transformed with the indicated expression vectors. β -galactosidase activity of mid-log phase cultures was quantitated as described previously (Johnson et al., 1986). β -galactosidase activity was measured for at least three independent transformants.

Transfection of *Drosophila* tissue culture cells.

Reporter constructs were all derived from pBLcat2 and have been previously described (Zamanian and La Thangue, 1992). Open and solid boxes denote wild-type and mutant E2F binding sites, respectively. pDP-1 encodes a complete DP-1 protein, and pG4mpolyII the Gal4 DNA binding domain (Webster et al., 1989). pE2F-1 has been previously described as pCMV RBAP-1 (Kaelin et al., 1992). Cells were transfected by the calcium phosphate procedure and harvested 40 to 45h later and for each transfection, pBluescript KS was included to maintain the final DNA concentration constant. All transfections included an internal control pCMV β -gal. The assay for CAT activity, correction for transfection efficiency and quantitation of TLC plates have been described previously (Zamanian and La Thangue, 1992).

Transfection of mammalian cells.

Reporter constructs were all derived from pBLcat2 and have been previously described (Zamanian and La Thangue, 1992). Open and solid boxes denote wild-type and mutant E2F binding sites, respectively. The plasmid pDP-1⁷³⁻³⁴⁰ encodes a protein spanning amino acids 73 to 340 in the wild-type DP-1 sequence fused downstream of the Gal4 sequences in pG4mpolyII (Webster et al., 1989). pCMV E2F-1 has been previously described as pCMV RBAP-1 (Kaelin et al., 1982). Cultivation of F9 EC cells and their transfection have been previously described (Zamanian and La Thangue, 1992). For each transfection, pBluescript KS was included to maintain the final DNA concentration constant. All transfections included an internal control pCMV β -gal. The assay for CAT activity and quantitation of TLC plates have been described previously (Zamanian and La Thangue, 1992).

Detailed description of the drawings.

Figure 6

DP-1 and E2F-1 exist in the same protein complex in vivo.

a) DP-1 is in DRTF1/E2F DNA binding complexes formed in HeLa cell extracts: gel retardation was performed using F9 EC and HeLa whole cell extracts (in which DRTF1 resolves as three distinct complexes, a, b, and c; indicated in figure) with the E2F binding site taken from the adenovirus E2A promoter (nucleotides -71 to -50) in the presence of either preimmune (PI; tracks 2 and 6) or immune (I; tracks 3 to 5 and 7 to 9) anti-DP-1 (peptide A) antiserum with the addition of either unrelated peptide 1 (tracks 4 and 8) or peptide A (tracks 5 and 9). In both F9 EC and HeLa cell extracts, all the DRTF1/E2F DNA binding complexes were affected by the anti-DP-1 antibody.

b) Anti-DP-1 immunoprecipitates DRTF1/E2F DNA binding activity: immunoprecipitation was performed from HeLa cell extracts with anti-DP-1 in the presence of either homologous peptide A (tracks 2 to 4) or unrelated peptide 1 (tracks 5 to 7). The immunoprecipitates were treated with 1% deoxycholate (DOC) and 1.5% NP40, and the detergent-released material assayed for DRTF1/E2F DNA binding activity; the depleted HeLa cell extract is also indicated (Sn; tracks 2 and 5). No DNA binding activity was released in the absence of detergent (indicated by c; tracks 3 and 6).

c) Immunoblotting DP-1 immunoprecipitates with anti-E2F-1: anti-DP-1 immunoprecipitates performed in the presence of either peptide A (track 3) or peptide 1 (track 4) were immunoblotted with the anti-E2F-1 monoclonal antibody SQ41; the E2F-1 polypeptide, present in track 4, is indicated by the arrow. As a positive control, about 100ng of the E2F-1 fusion protein, GST-E2F-1⁸⁹⁻⁴³⁷, was immunoblotted in track 2. Track 1 shows standard molecular weights.

Figure 7

DP-1 and E2F-1 bind to the E2F site as a complex.

a) DP-1 and E2F-1 interact synergistically in DNA binding to the E2F site: GST-DP-1⁵⁹⁻⁴¹⁰ (about 25ng) or GST-E2F-1⁸⁹⁻⁴³⁷ (about 50ng)

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were assayed either alone (tracks 2, 3, 6 and 7) or together (tracks 4 and 8) for binding to the adenovirus E2A promoter (tracks 1 to 4) or the distal E2F site taken from the E2A promoter (tracks 5 to 8); tracks 1 and 5 show the binding sites alone. Note that a DNA binding complex was apparent in track 6 upon increased exposure (data not shown). The E2F site-specificity of the complexes was confirmed by performing the appropriate competition experiments (data not shown). The effect of anti-E2F-1 (tracks 9 and 10) or anti-DP-1 (tracks 11 and 12; anti-peptide 18; Girling et al., 1993) was assessed on GST-E2F-1⁸⁹⁻⁴³⁷ alone (track 9) or GST-E2F-1⁸⁹⁻⁴³⁷ and GST-DP-1⁵⁹⁻⁴¹⁰ together (tracks 10, 11 and 12). In addition, the reactions in tracks 11 and 12 contain either an unrelated (track 11) or the homologous (peptide 18; track 12) peptides.

b) DP-1 and E2F-1 form DNA binding heteromers: GST-E2F-1⁸⁹⁻⁴³⁷ (about 50ng) was incubated with control GST fusion protein (about 300ng; track 1) or DP-1⁸⁴⁻²⁴⁹ or DP-1⁸⁴⁻²⁰⁴ (about 150ng, released after cleavage with thrombin; tracks 2 and 3), GST-DP-1⁸⁴⁻²⁴⁹, GST-DP-1⁸⁴⁻²⁰⁴, GST-DP-1¹⁴⁶⁻²⁴⁹ or GST-DP-1⁸⁴⁻¹⁶⁶ (about 300ng, without cleavage; tracks 4, 5, 6 and 7).

c) Sequence specificity of the E2F-1⁸⁹⁻⁴³⁷/DP-1⁸⁴⁻²⁴⁹ heteromer: the DNA sequence specificity of complexes formed by either GST-E2F-1⁸⁹⁻⁴³⁷ (50ng; tracks 2 to 6) or GST-E2F-1⁸⁹⁻⁴³⁷ with DP-1⁸⁴⁻²⁴⁹ (50ng and 150ng respectively; tracks 7 to 11) was determined by competing with the wild-type or mutated derivatives of the distal E2F site from the adenovirus E2A promoter (about 100-fold molar excess of the binding sites indicated). For comparison, a similar experiment is shown in an F9 EC cell extract (track 12 to 16). Both mono- and heteromeric DNA binding complexes had very similar sequence specificities to F9 EC cell DRTF1/E2F. Track 1 shows the probe alone. Details of the competing binding sites are given in Materials and Methods.

d) DP-1 contains a dimerization domain: the indicated regions of DP-1 were expressed as GST fusion proteins (tracks 3 to 6) and about 2µg incubated with 5µl of a reticulocyte lysate containing translated wild-type E2F-1¹⁻⁴³⁷. GST fusion proteins, or GST

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protein alone (track 2), were collected with glutathione-agarose beads and bound E2F-1 polypeptide released. Track 1 shows the lysate with the E2F-1 polypeptide. Note that DP-1¹⁴⁶⁻²⁴⁹ binds to E2F-1.

e) Summary of the data and molecular properties of DP-1. The C-terminal border of the DNA binding domain, which is known to lie within the region indicated by the broken line, has not been defined.

Figure 8

DP-1 and E2F-1 interact in yeast cells.

Summary of results. Details of the expression vectors and reporter construct are described above.

Figure 9

Functional synergy between DP-1 and E2F-1 in *Drosophila* SL2 cells

a) Summary of constructs: p3xWT and p3xMT have been previously described (Zamanaian and La Thangue, 1992). pDP-1 and pE2F-1 contain full length proteins, and pG4MpolyII the Gal4 DNA binding domain.

b) and c). SL2 cells were transfected with p3xWT and the indicated expression vectors. The amounts of expression vector in each treatment were as follows: 50ng (lanes 1, 5, 6, 7 and 8) or 500ng (lane 2) for E2F-1, 5µg (lanes 3 and 5) or 10µg (lanes 4 and 6) for DP-1, and 3.7µg (lane 7) or 7.0µg (lane 8) for pG4Mpoly II. All values are expressed relative to p3xWT alone which was given an arbitrary value of 1.0, and are representative of at least three separate experiments. b) shows an example of the crude data which is quantitatively represented in c).

Figure 10

DP-1 contributes to E2F site dependent transcription in F9 EC cells.

a) Summary of constructs

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b) F9 EC cells were transfected with p3xWT and the indicated expression vectors. All treatments were performed in duplicate and corrected for transfection efficiency. All values are expressed relative to the activity of p3xWT alone which was given an arbitrary value of 1.0, and are representative of at least three separate experiments. Note that DP-1⁷³⁻³⁴⁰ cripples the endogenous DRTF1/E2F activity and that E2F-1 can rescue this effect. The data are represented graphically at the bottom of the figure.

Figure 11

DP-1 and E2F-1 activate E2F site-dependent transcription in yeast cells.

- a) Summary of constructs.
- b) β -galactosidase activity was measured in *S.cerevisiae* strain W3031a carrying p4xWT CYC1 and the indicated effector expression vector. All values are expressed relative to the activity of p4xWT CYC1 which was given an arbitrary value of 1.0 and are representative of at least three separate experiments.

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389-396

- 47 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Medical Research Council
- (B) STREET: 20 Park Crescent
- (C) CITY: London
- (D) STATE: London
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): W1N 4AL

(ii) TITLE OF INVENTION: Transcription Factor

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0; Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1700 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 55..1284

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Met	
1	
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Ala Lys Asp Ala Ser Leu Ile Glu Ala Asn Gly Glu Leu Lys Val Phe	
5 10 15	
ATA GAC CAG AAT CTT AGT CCT GGG AAA GGT GTG GTA TCT CTT GTA GCC	153
Ile Asp Gln Asn Leu Ser Pro Gly Lys Gly Val Val Ser Leu Val Ala	
20 25 30	
GTC CAC CCG TCC ACA GTC AAC ACA CTT GGG AAG CAG CTT TTG CCA AAA	201
Val His Pro Ser Thr Val Asn Thr Leu Gly Lys Gln Leu Leu Pro Lys	
35 40 45	
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Thr Phe Gly Gln Ser Asn Val Asn Ile Thr Gln Gln Val Val Ile Gly	
50 55 60 65	
ACG CCT CAG AGA CCG GCA GCA TCC AAC ACT ATT GTG GTA GGA AGC CCA	297
Thr Pro Gln Arg Pro Ala Ala Ser Asn Thr Ile Val Val Gly Ser Pro	
70 75 80	
CAC ACT CCC AAC ACG CAT TTT GTG TCA CAG AAC CAG ACG TCT GAC TCC	345
His Thr Pro Asn Thr His Phe Val Ser Gln Asn Gln Thr Ser Asp Ser	
85 90 95	

- 48 -

TCA CCT TGG TCT GCT GGG AAG CGG AAC AGG AAG GGC GAG AAG AAT GGC Ser Pro Trp Ser Ala Gly Lys Arg Asn Arg Lys Gly Glu Lys Asn Gly 100 105 110	393
AAG GGC CTG CGG CAT TTC TCC ATG AAG GTG TGT GAG AAG GTG CAG AGG Lys Gly Leu Arg His Phe Ser Met Lys Val Cys Glu Lys Val Gln Arg 115 120 125	441
AAA GGA ACC ACC TCC TAC AAT GAG GTG GCT GAC GAG CTG GTG GCA GAG Lys Gly Thr Thr Ser Tyr Asn Glu Val Ala Asp Glu Leu Val Ala Glu 130 135 140 145	489
TTC AGC GCT GCC GAC AAC CAC ATT CTA CCA AAC GAA TCA GCT TAT GAC Phe Ser Ala Ala Asp Asn His Ile Leu Pro Asn Glu Ser Ala Tyr Asp 150 155 160	537
CAG AAG AAC ATC CGG CGG CGT GTC TAC GAT GCC TTA AAT GTG CTA ATG Gln Lys Asn Ile Arg Arg Arg Val Tyr Asp Ala Leu Asn Val Leu Met 165 170 175	585
GCC ATG AAC ATC ATC TCC AAG GAG AAG AAG GAG ATC AAA TGG ATC GGC Ala Met Asn Ile Ile Ser Lys Glu Lys Lys Glu Ile Lys Trp Ile Gly 180 185 190	633
CTG CCC ACC AAC TCA GCT CAG GAG TGC CAG AAC TTA GAG GTG GAG AGG Leu Pro Thr Asn Ser Ala Gln Glu Cys Gln Asn Leu Glu Val Glu Arg 195 200 205	681
CAG AGG AGG CTG GAG AGG ATC AAA CAG AAG CAG TCT CAG CTC CAG GAG Gln Arg Arg Leu Glu Arg Ile Lys Gln Lys Gln Ser Gln Leu Gln Glu 210 215 220 225	729
CTC ATC CTG CAG CAA ATT GCC TTC AAG AAC TTG GTG CAG AGA AAT CGC Leu Ile Leu Gln Gln Ile Ala Phe Lys Asn Leu Val Gln Arg Asn Arg 230 235 240	777
CAA GCT GAG CAG CAG GCC CGC AGG CCG CCT CCT CCC AAC TCT GTC ATC Gln Ala Glu Gln Gln Ala Arg Arg Pro Pro Pro Pro Asn Ser Val Ile 245 250 255	825
CAC TTG CCC TTC ATC ATT GTC AAC ACC AGC AGG AAG ACA GTC ATT GAC His Leu Pro Phe Ile Ile Val Asn Thr Ser Arg Lys Thr Val Ile Asp 260 265 270	873
TGC AGC ATC TCC AAT GAC AAA TTT GAG TAT CTG TTT AAC TTT GAC AAC Cys Ser Ile Ser Asn Asp Lys Phe Glu Tyr Leu Phe Asn Phe Asp Asn 275 280 285	921
ACG TTT GAG ATC CAC GAT GAC ATT GAG GTG CTC AAG CGC ATG GGC ATG Thr Phe Glu Ile His Asp Asp Ile Glu Val Leu Lys Arg Met Gly Met 290 295 300 305	969
GCA TGT GGG CTG GAG TCT GGC AAC TGC TCT GCT GAA GAC CTC AAG GTG Ala Cys Gly Leu Glu Ser Gly Asn Cys Ser Ala Glu Asp Leu Lys Val 310 315 320	1017
GCC AGA AGT TTG GTA CCA AAA GCT CTA GAA CCA TAC GTG ACA GAA ATG Ala Arg Ser Leu Val Pro Lys Ala Leu Glu Pro Tyr Val Thr Glu Met 325 330 335	1065
GCT CAG GGA TCC ATT GGT GGC GTA TTC GTC ACG ACA ACA GGT TCT ACA Ala Gln Gly Ser Ile Gly Gly Val Phe Val Thr Thr Thr Gly Ser Thr 340 345 350	1113
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- 49 -

GGG ATG CTG GCC ACG AGC TCC AAT GGG TCT CAG TAC AGC GGC TCC AGG 1209
 Gly Met Leu Ala Thr Ser Ser Asn Gly Ser Gln Tyr Ser Gly Ser Arg
 370 375 380 385

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 Val Glu Thr Pro Val Ser Tyr Val Gly Glu Asp Asp Asp Asp Asp Asp
 390 395 400

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 Asp Phe Asn Glu Asn Asp Glu Glu Asp
 405 410

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GTGCAGCTCC AAGTGAGGAG GAGTGTGCGC AGTTTGAGCC TAGCTGCGGA TGTGTTGTGA 1484

AGCCAGCGTG CTAATGCAGA GCCTCTATCT ACCTTTTAGG ATTTTATGGT TTCTCTCTTT 1544

TCTCTCTTTT TTTTCCTTTT CTTTCTTTTT TGAGTTTGAA GCTTATTTTG CCCCTCAACA 1604

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GCTGCCCTGC CTCCGATGTC CAGCACCCAG GTGGTG 1700

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 410 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Phe Ile Asp Gln Asn Leu Ser Pro Gly Lys Gly Val Val Ser Leu Val
 20 25 30

Ala Val His Pro Ser Thr Val Asn Thr Leu Gly Lys Gln Leu Leu Pro
 35 40 45

Lys Thr Phe Gly Gln Ser Asn Val Asn Ile Thr Gln Gln Val Val Ile
 50 55 60

Gly Thr Pro Gln Arg Pro Ala Ala Ser Asn Thr Ile Val Val Gly Ser
 65 70 75 80

Pro His Thr Pro Asn Thr His Phe Val Ser Gln Asn Gln Thr Ser Asp
 85 90 95

Ser Ser Pro Trp Ser Ala Gly Lys Arg Asn Arg Lys Gly Glu Lys Asn
 100 105 110

Gly Lys Gly Leu Arg His Phe Ser Met Lys Val Cys Glu Lys Val Gln
 115 120 125

Arg Lys Gly Thr Thr Ser Tyr Asn Glu Val Ala Asp Glu Leu Val Ala
 130 135 140

Glu Phe Ser Ala Ala Asp Asn His Ile Leu Pro Asn Glu Ser Ala Tyr
 145 150 155 160

Asp Gln Lys Asn Ile Arg Arg Arg Val Tyr Asp Ala Leu Asn Val Leu
 165 170 175

- 50 -

Met Ala Met Asn Ile Ile Ser Lys Glu Lys Lys Glu Ile Lys Trp Ile
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 Gly Leu Pro Thr Asn Ser Ala Gln Glu Cys Gln Asn Leu Glu Val Glu
 195 200 205
 Arg Gln Arg Arg Leu Glu Arg Ile Lys Gln Lys Gln Ser Gln Leu Gln
 210 215 220
 Glu Leu Ile Leu Gln Gln Ile Ala Phe Lys Asn Leu Val Gln Arg Asn
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 Arg Gln Ala Glu Gln Gln Ala Arg Arg Pro Pro Pro Pro Asn Ser Val
 245 250 255
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 260 265 270
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 305 310 315 320
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 325 330 335
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 370 375 380
 Arg Val Glu Thr Pro Val Ser Tyr Val Gly Glu Asp Asp Asp Asp Asp
 385 390 395 400
 Asp Asp Phe Asn Glu Asn Asp Glu Glu Asp
 405 410

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CLAIMS

1. A polynucleotide in substantially isolated form which comprises a contiguous sequence of nucleotides which is capable of selectively hybridizing to Seq. ID No. 1 or to the complement of Seq. ID No. 1.
2. A polynucleotide according to claim 1 which is a DNA polynucleotide.
3. A polynucleotide according to claim 1 or 2 wherein the contiguous sequence comprises at least 20 nucleotides.
4. A polynucleotide according to claim 3 which comprises Seq. ID No. 1.
5. A double stranded polynucleotide comprising a polynucleotide according to any one of claims 1 to 4 and its complement.
6. A polynucleotide according to any one of claims 1 to 5 carrying a revealing label.
7. A recombinant replicable vector which comprises a polynucleotide according to any one of claims 1 to 6.
8. A host cell comprising a vector according to claim 7.
9. A polypeptide in substantially isolated form which comprises:
 - (a) the protein of Seq. ID No. 2; or
 - (b) an allelic variant or species homologue thereof; or
 - (c) a protein at least 70% homologous to (a); or
 - (d) a fragment of any one of (a) to (c) capable of forming a complex with the E2F-1 protein or related family member; or
 - (e) a fragment of any one of (a) to (c) of at least 15 amino acids.

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10. A polypeptide according to claim 9 carrying a revealing label.
11. A composition comprising a polypeptide according to claim 9 or 10 together with a carrier or a diluent.
12. A polypeptide according to claim 9 or 10 fixed to a solid phase.
13. A double stranded DNA encoding a polypeptide as defined in claim 9.
14. A recombinant replicable vector comprising a coding sequence which encodes a polypeptide as defined in claim 9.
15. A host cell transformed by a recombinant vector according to claim 14.
16. A host cell transformed by a recombinant vector according to claim 14 wherein the coding sequence is operably linked to a control sequence capable of providing for the expression of the coding sequence by the host cell.
17. A process for preparing a polypeptide according to claim 9 which comprises cultivating a host cell according to claim 16 under conditions to provide for expression by the recombinant vector of the coding sequence, and recovering the expressed polypeptide.
18. An antibody capable of binding:
 - (a) the protein of Seq. ID No. 2; or
 - (b) an allelic variant or species homologue thereof; or
 - (c) a protein at least 70% homologous to (a); or
 - (d) a fragment of any one of (a) to (c) capable of forming a complex with the E2F-1 protein or related family member; or
 - (e) a fragment of any one of (a) to (c) of at least 15 amino acids.

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19. An antibody according to claim 18 which is a monoclonal antibody.
20. An antibody according to claim 18 or 19 carrying a revealing label.
21. An antibody according to any one of claims 18 to 20 fixed to a solid phase.
22. A hybridoma cell line which produces a monoclonal antibody according to claim 19.
23. An immunoassay for detecting the presence or absence of a polypeptide according to claim 9 in a sample which comprises:
 - (a) providing an antibody according to any one of claims 18 to 21;
 - (b) incubating the sample with said antibody under conditions that allow for the formation of an antibody-antigen complex; and
 - (c) detecting said antibody-antigen complex.
24. A screening method for identifying putative chemotherapeutic agents for the treatment of proliferative or viral disease which comprises
 - (A) bringing into contact:
 - (i) a polypeptide according to claim 9 or 10;
 - (ii)
 - (a) the E2F-1 protein, or
 - (b) an allelic variant or species homologue thereof, or
 - (c) an E2F-1 family member having at least 70% homology over the DNA binding region, or
 - (d) a fragment of (a), (b) or (c) capable of forming a functional trans-activation complex with the protein of Seq. ID No. 2; or
 - (e) a fusion protein comprising (a), (b), (c), or (d); and
 - (iii) a putative chemotherapeutic agent;

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under conditions in which the components (i) and (ii) in the absence of (iii) form a complex, and

(B) measuring the extent to which component (iii) is able to disrupt or inhibit the activity of said complex.

25. An assay according to claim 23 wherein the complex of (i) and (ii) is measured by its ability to bind an E2F DNA binding site in vitro.

26. An assay according to claim 23 wherein the complex of (i) and (ii) is measured by its ability to activate in vivo a promoter comprising an E2F binding site linked to a reporter gene.

27. An assay according to claim 26 wherein the assay is performed in a yeast cell, insect cell or a mammalian cell.

28. An assay according to any one of claims 23 to 26 wherein the putative chemotherapeutic agent is a fragment of 10 or more amino acids of:

- (a) the protein of Seq. ID No. 2; or
- (b) an allelic variant or mammalian homologue thereof;
or
- (c) a protein at least 70% homologous to (a).

29. An assay according to claim 28 wherein the fragment is a polypeptide fragment falling within the region of 160 to 220 of Seq. ID No. 2.

30. A polypeptide according to claim 9 or 10 or an antibody according to any one of claims 18 to 20 for use in a method of treatment of the human or animal body.

31. A vector according to claim 7 or 14 for use in a method of treatment of the human or animal body.

32. A method of treating uncontrolled proliferation of cells in a patient in need of treatment which comprises administering to the patient an effective amount of a vector according to claim 7 or 14, or a polynucleotide according to claim 1.

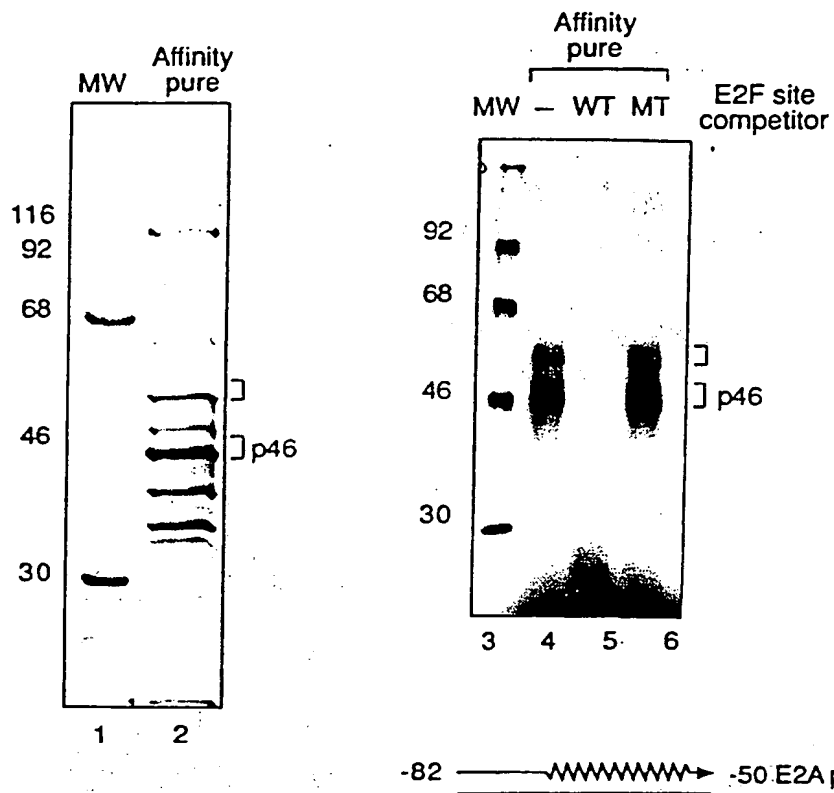
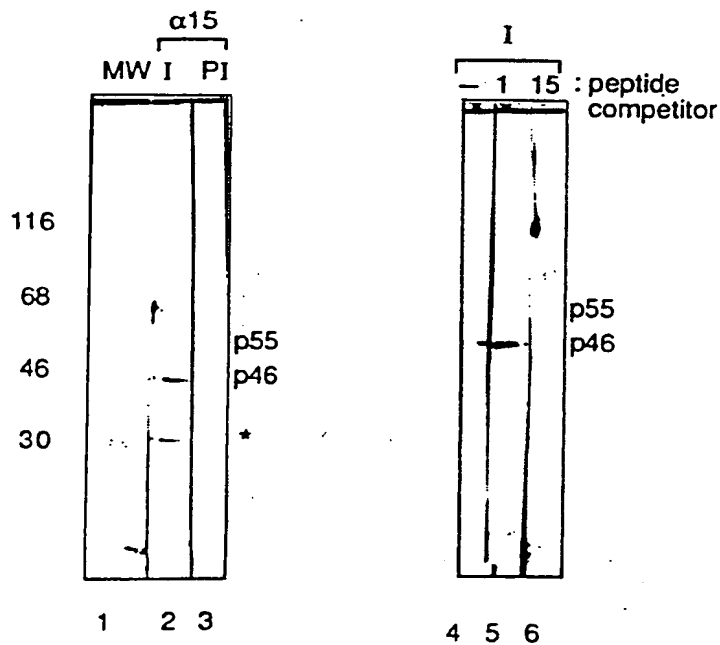
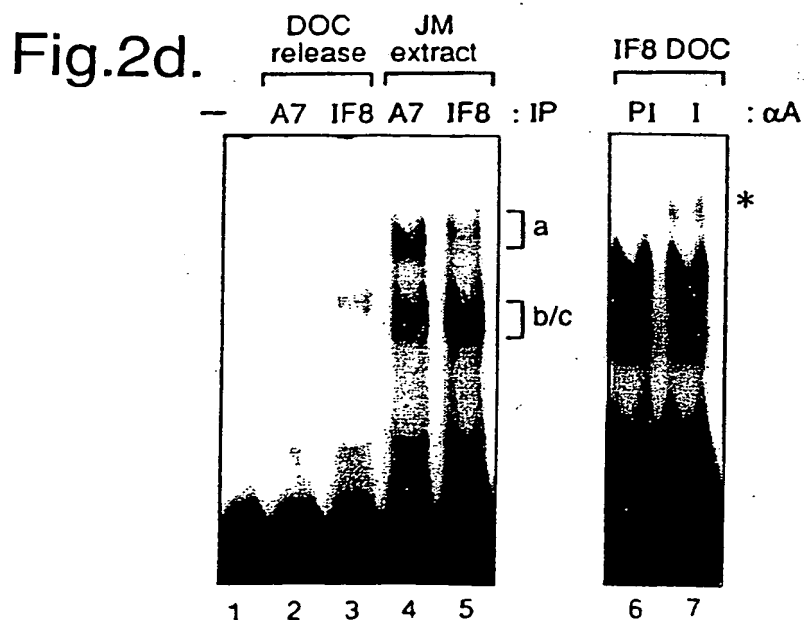
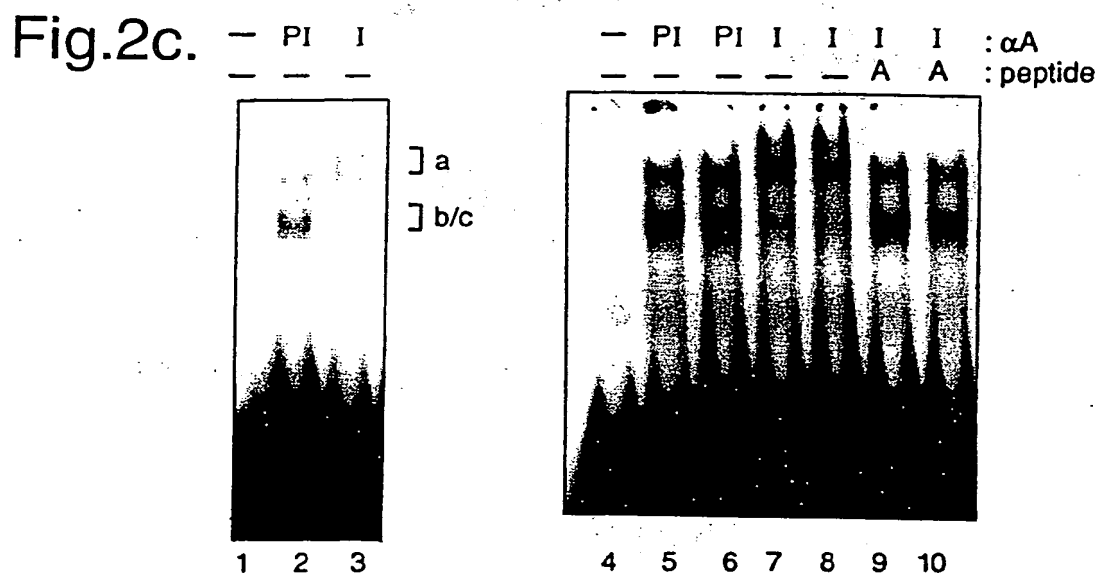
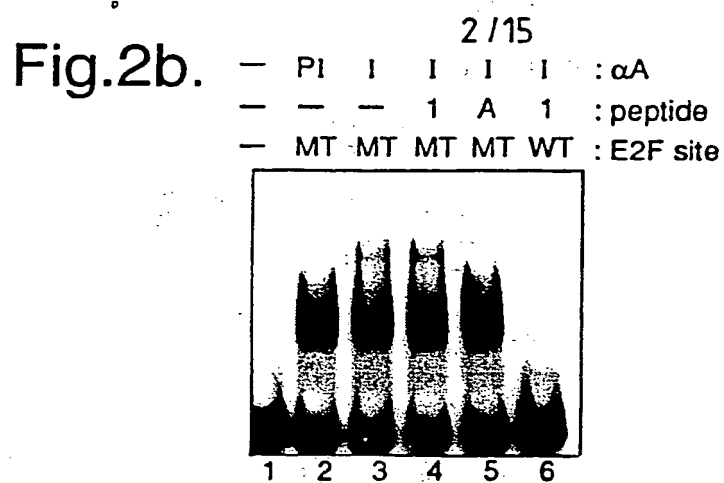
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Fig.1.

Fig.2a.



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Fig.3a.

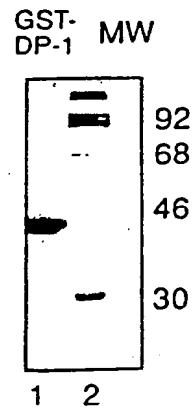
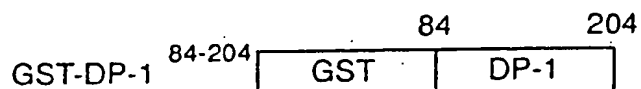
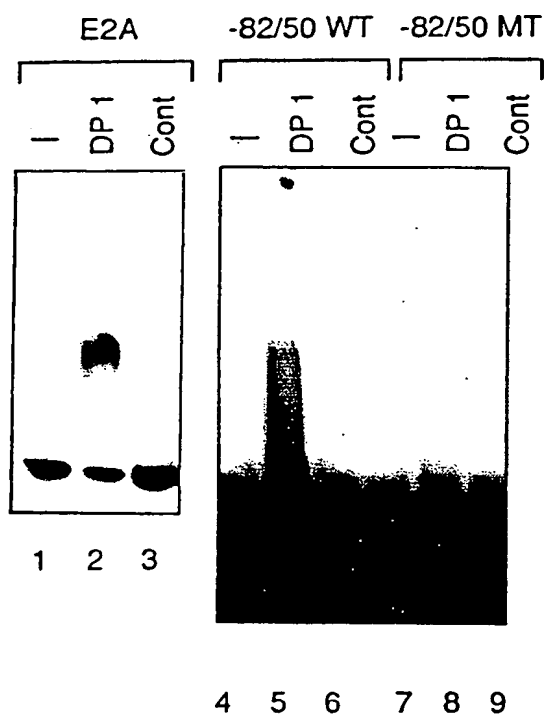


Fig.3b.



-82 ————— -50 WT
-82 ————— *** -50 MT
-62/60

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Fig.3c.

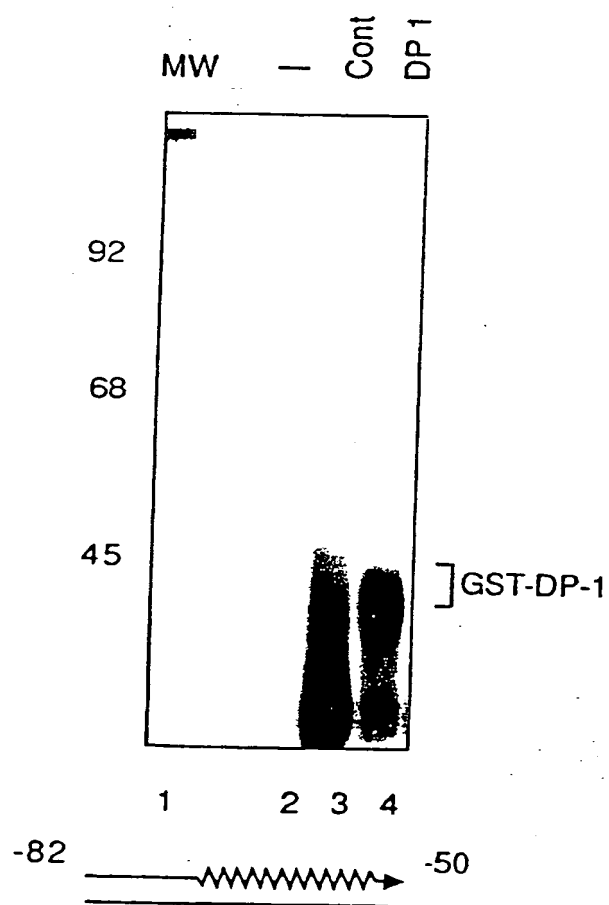
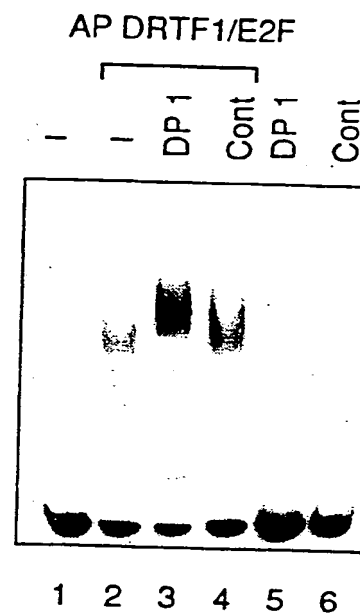


Fig.3d.



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Fig.4a.

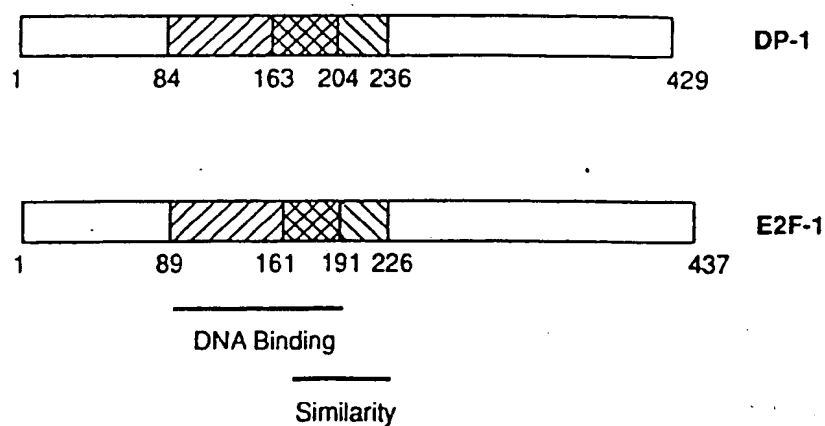


Fig.4b.

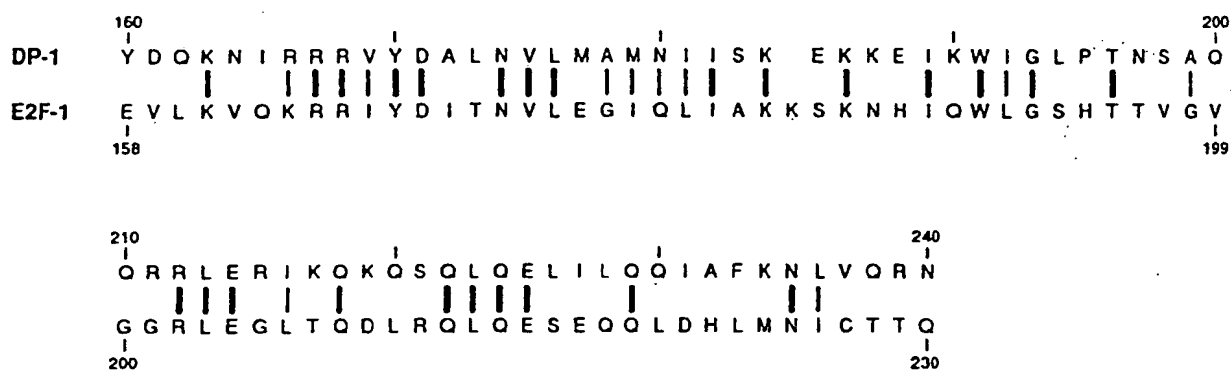
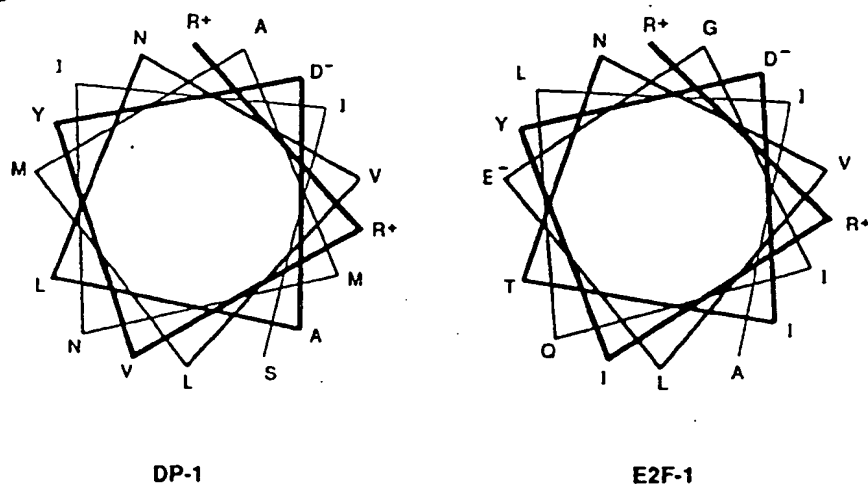


Fig.4c.



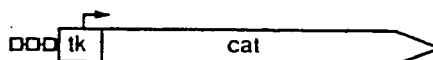
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Fig.5a.

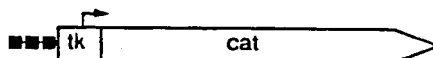
Summary of Constructs

Reporters

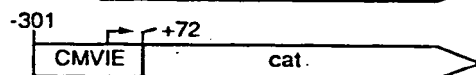
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p3xMT



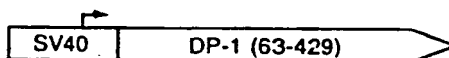
pCMVcat

Effectors

pG4mpoly II



pGB9



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Fig.5b.

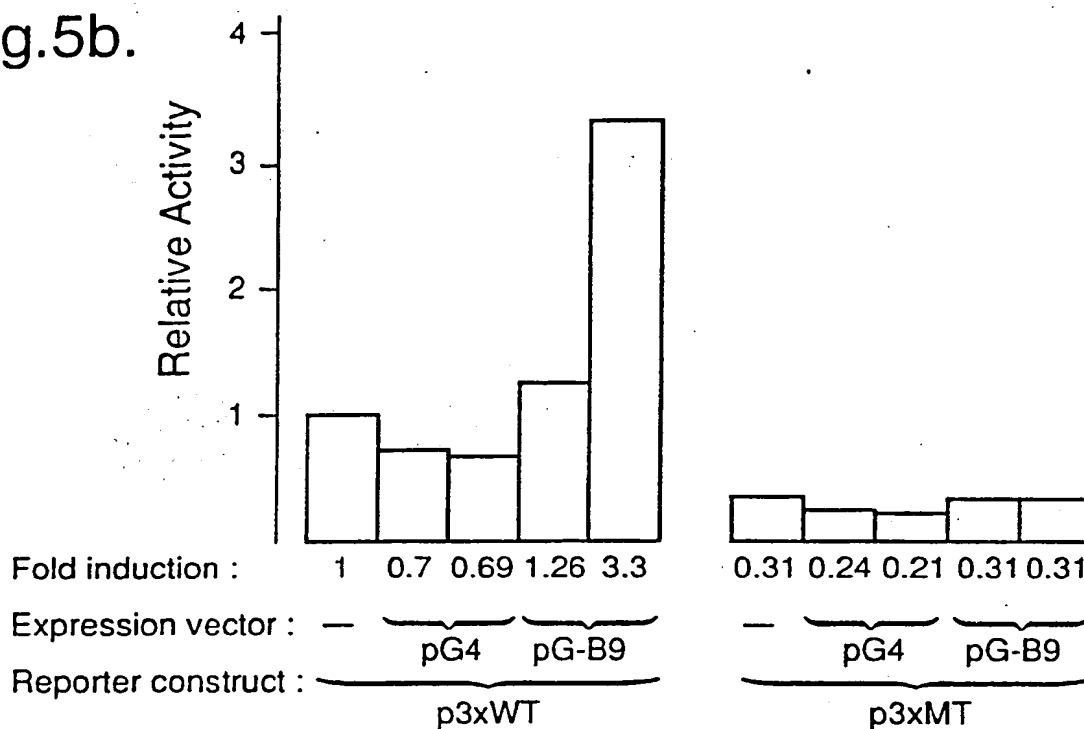
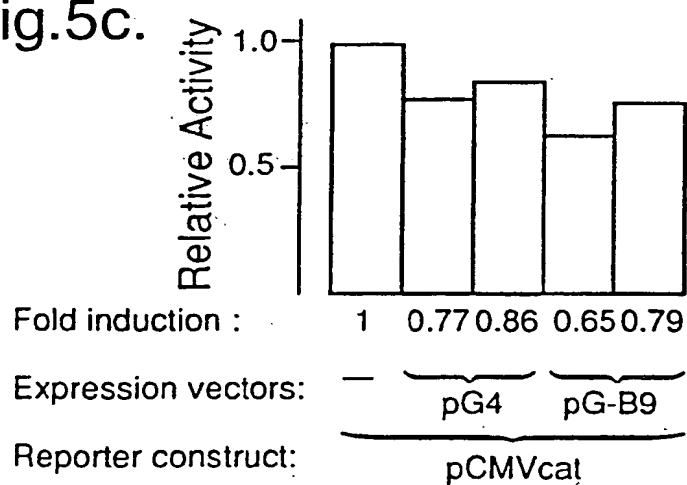


Fig.5c.



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Fig.6(a).

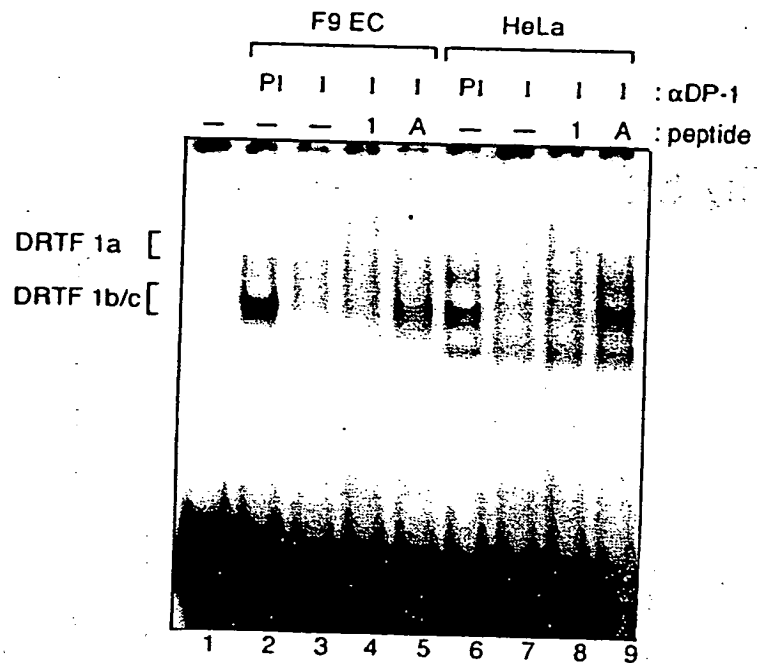


Fig.6(b).

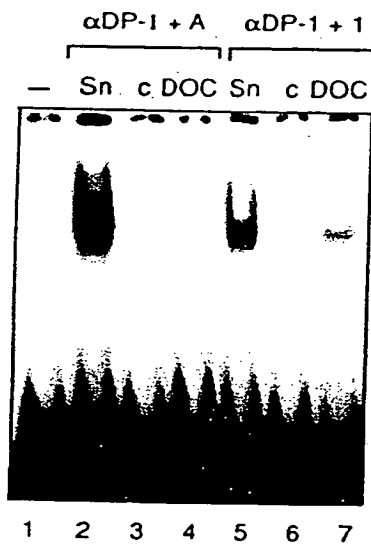
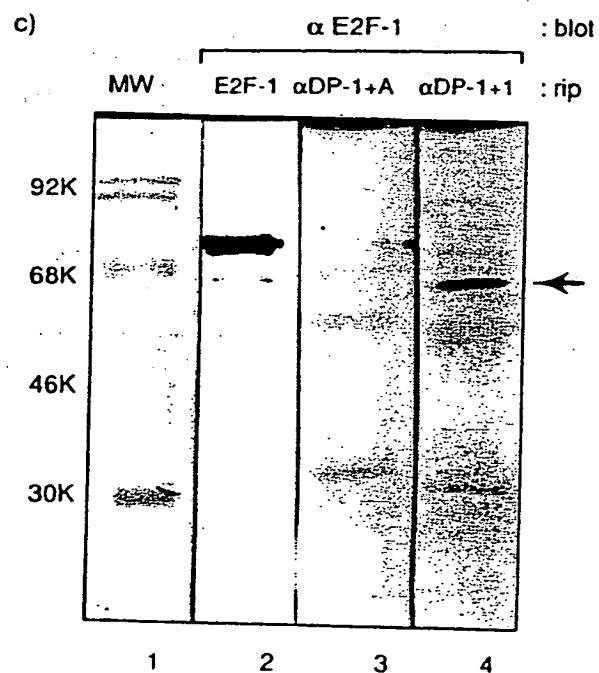


Fig.6(c).



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Fig.7(a).

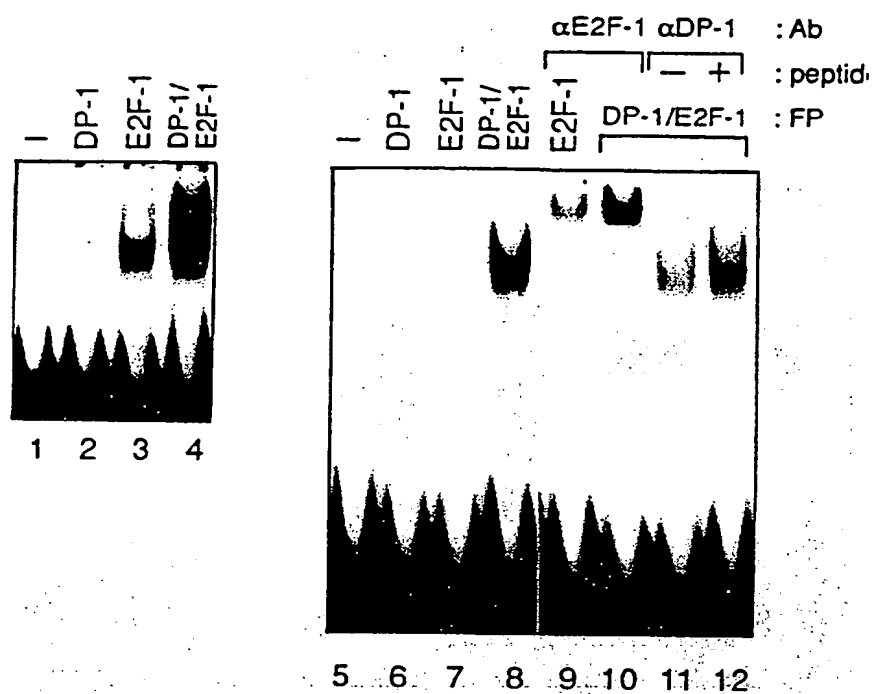
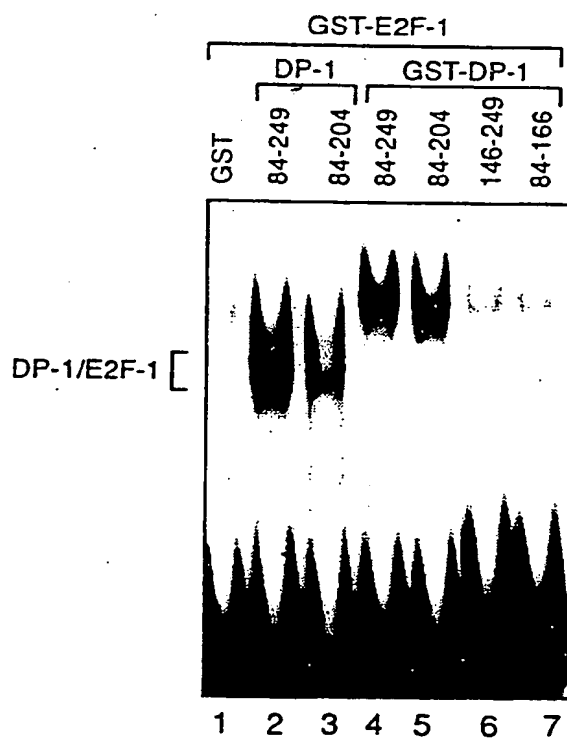


Fig.7(b).



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Fig.7(c).

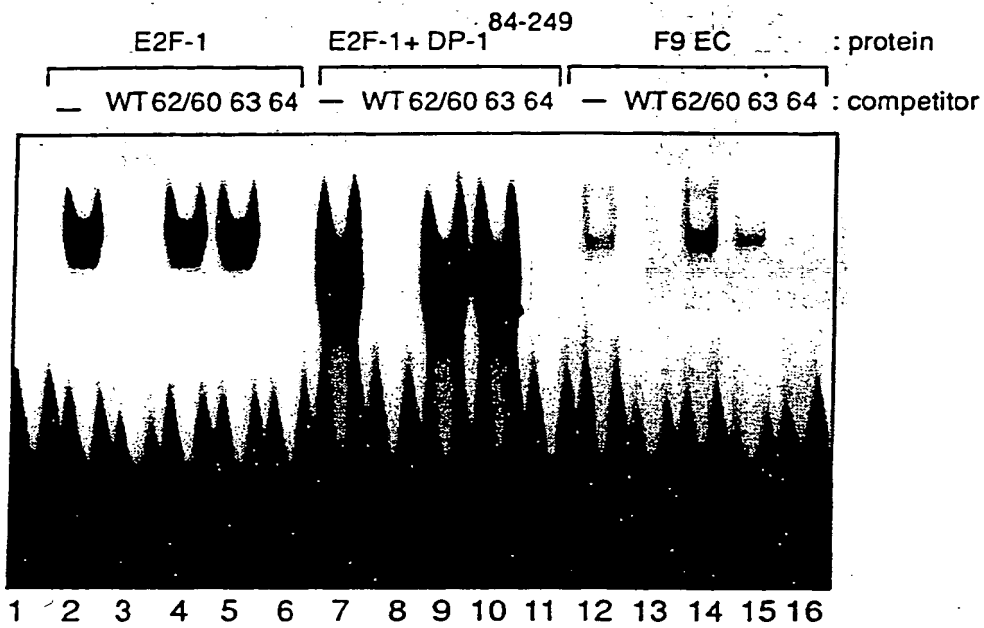
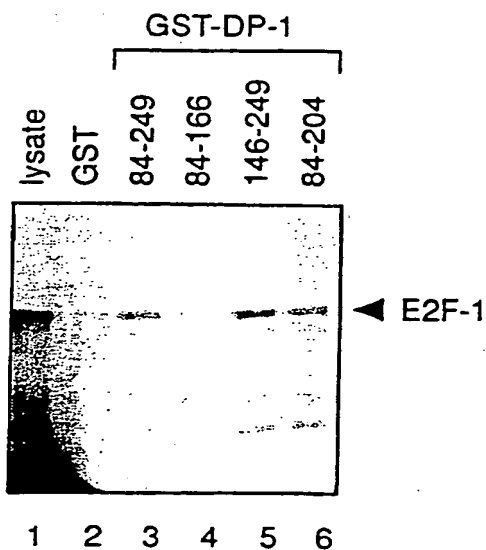


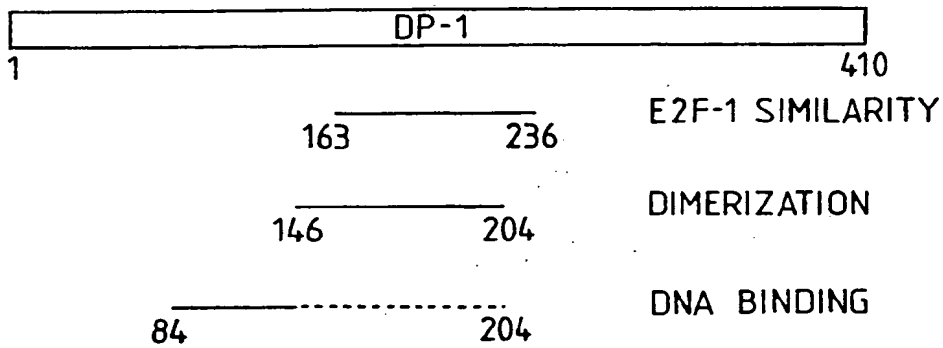
Fig.7(d).



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Fig.7(e).

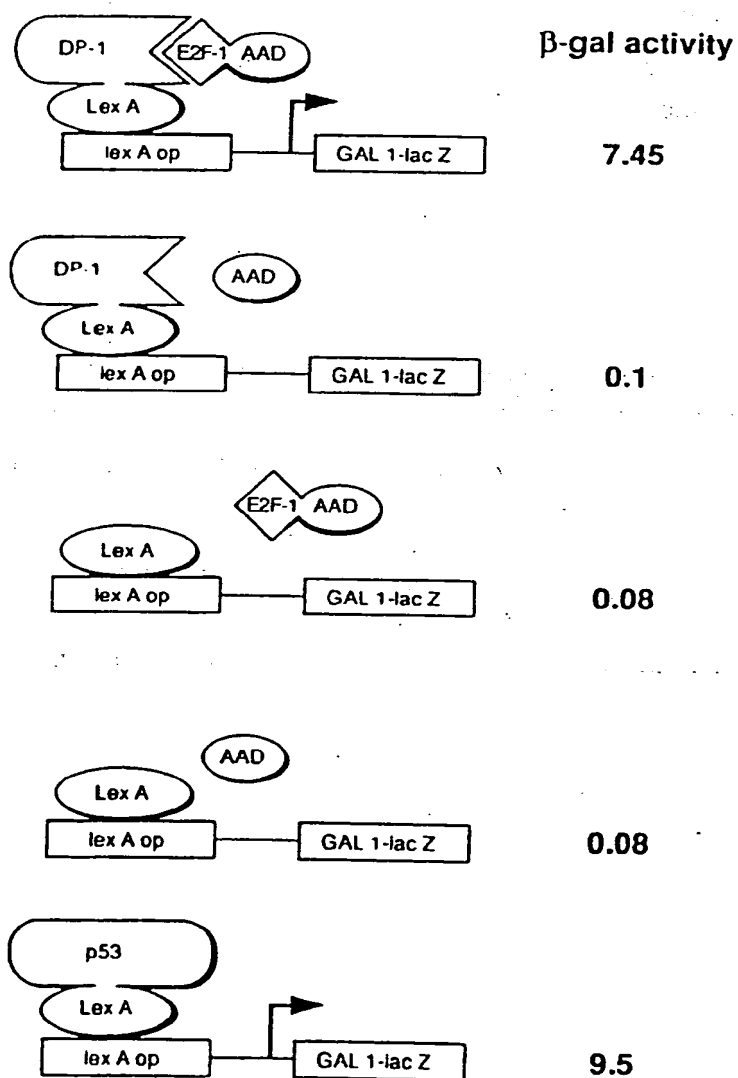
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	DNA BINDING	DIMERIZATION
DP-1 84-249	++	+
DP-1 84-204	+	+
DP-1 146-249	-	+
DP-1 84-166	-	-



- SUBSTITUTE SHEET

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Fig.8.



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Fig.9(a).

Reporters:

p3×WT

p3×MT

Effectors:

pDP-1

pE2F-1

pG4mpoly II

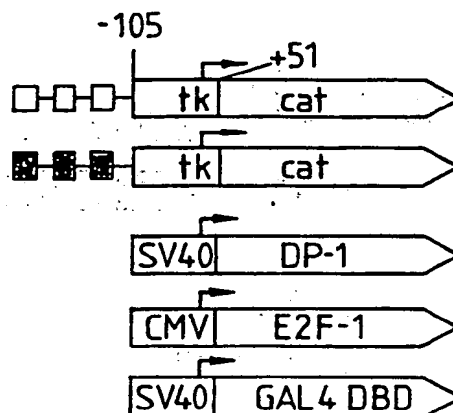


Fig.9(b).

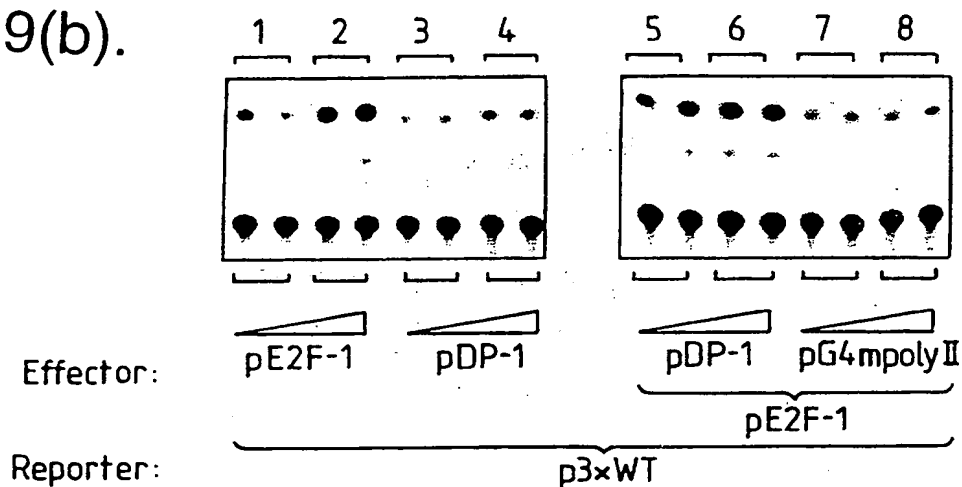
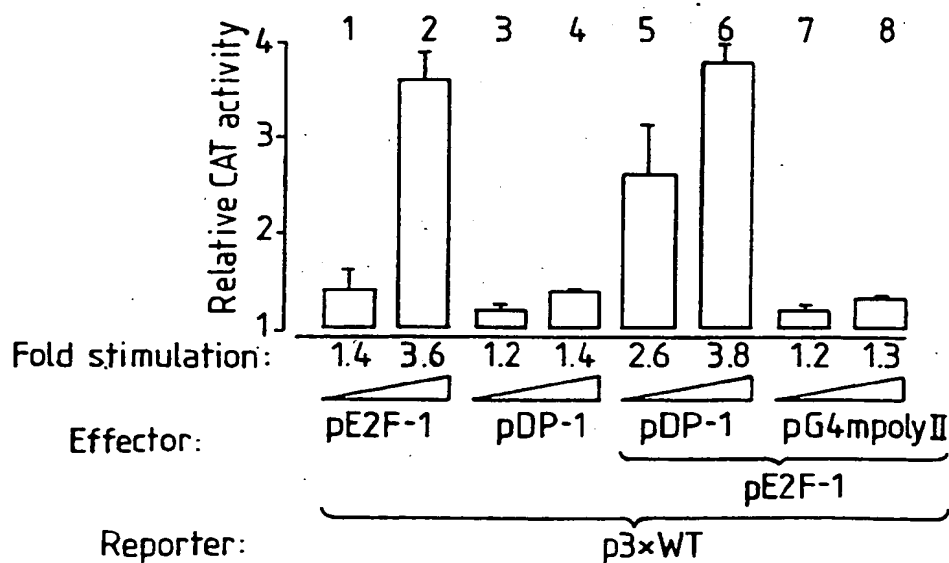
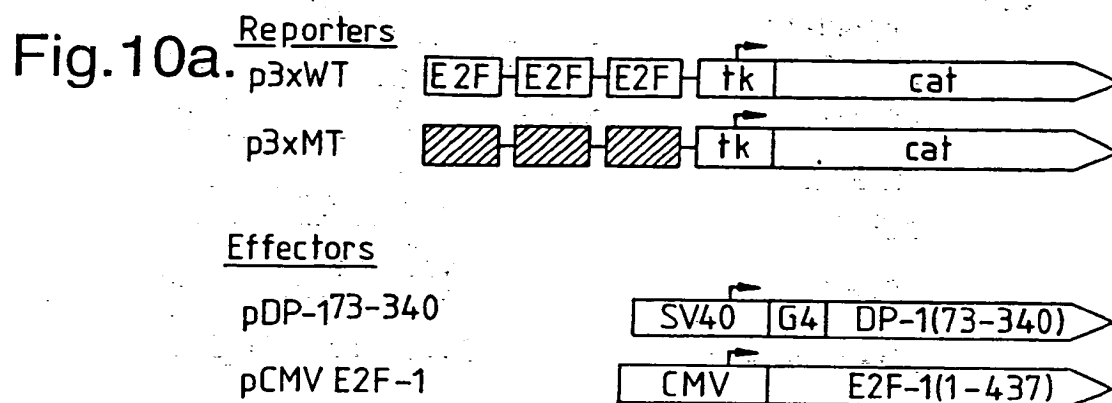
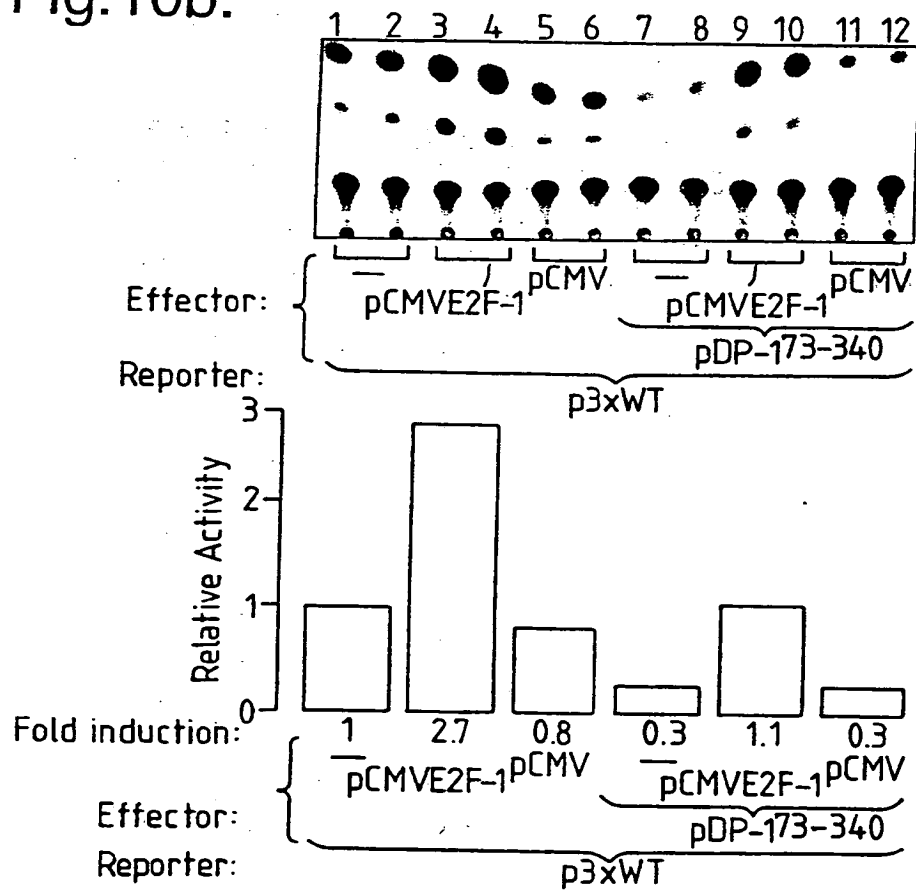


Fig.9(c).



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**Fig.10b.**

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Fig.11(a).

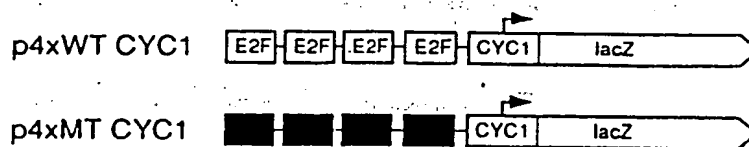
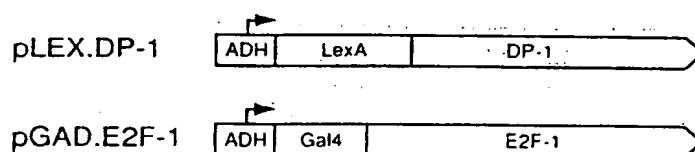
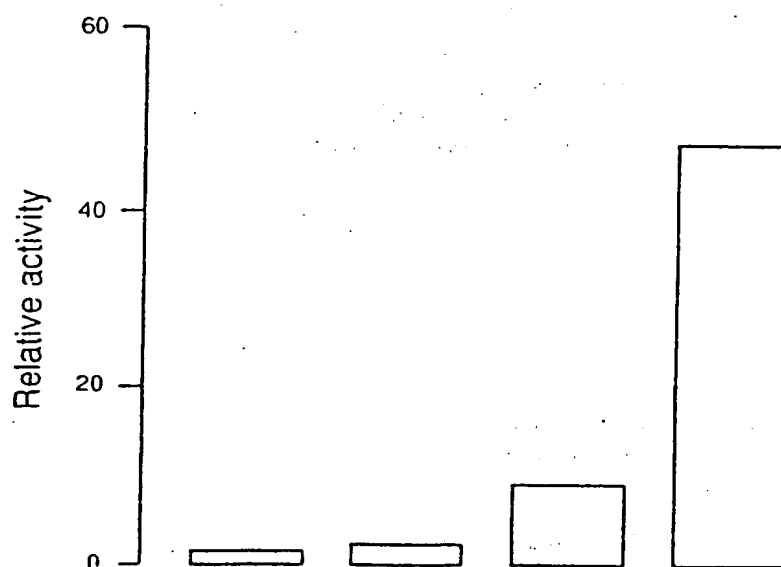
ReportersEffectors

Fig.11(b).



Fold Induction : 1 1.59 9.62 48.1

Effector : DP-1 E2F-1 DP-1/E2F-1

Reporter :

p4xWT CYC 1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/02227

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/12 C12N15/62 C12N15/67 C12N15/85 C07K13/00
C07K15/28 C12Q1/02 C12Q1/68 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C12Q C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EMBO JOURNAL vol. 11, no. 7, July 1992, IRL PRESS LIM., OXFORD, ENGL.; pages 2603 - 2610 M. ZAMANIAN AND N.B. LA THANGUE 'Adenovirus Ela prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor' cited in the application see page 2608, right column, paragraph 2 - page 2609, right column, line 37.</p> <p style="text-align: center;">-/--</p>	1-32

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- *Z* document member of the same patent family

Date of the actual completion of the international search

28 February 1994

Date of mailing of the international search report

07 -04- 1994

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INTERNATIONAL SEARCH REPORT

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CELL vol. 70, no. 2, 24 July 1992, CELL PRESS, CAMBRIDGE, MA, US; pages 351 - 364 W.G. KAEIN ET AL. 'Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties' cited in the application see page 358, right column, paragraph 3 - page 361, right column, paragraph 2 ---</p>	1-32
A	<p>MOL. CELL. BIOL. vol. 11, no. 3, March 1991, AM. SOC. MICROBIOL., WASHINGTON, D.C. US; pages 1686 - 1695 M.K.K. SHIVJI AND N.B. LA THANGUE 'Multicomponent differentiation-regulated transcription factors in F9 embryonal carcinoma stem cells' cited in the application see page 1687, left column, line 58 - page 1693, left column, line 30 ---</p>	1-19
P,X	<p>NATURE vol. 362, 4 March 1993, MACMILLAN JOURNALS LTD., LONDON, UK; pages 83 - 87 R. GIRLING ET AL. 'A new component of the transcription factor DRTF1/E2F' cited in the application see page 83, right column, line 1 - page 87, left column, line 60; figure 1 ---</p>	1-19
P,X	<p>WO,A,93 15227 (DUKE UNIVERSITY) 5 August 1993 see page 8, line 9 - line 4; claims 31,33-35 see page 9, line 1 - line 30 ---</p>	24-27
P,X	<p>CURRENT BIOLOGY vol. 3, no. 8, August 1993, LONDON, GB; pages 554 - 557 N.B. LA THANGUE AND L.H. JOHNSTON 'Transcriptional complexity' see page 554, left column, line 1 - page 556, right column, line 24 ---</p>	1-17
-/--		

INTERNATIONAL SEARCH REPORT

Internatic. application No

PCT/GB 93/02227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>GENES & DEVELOPMENT vol. 7, no. 10 , October 1993 , CSH LABORATORY PRESS, CSH, N.Y., US; pages 1850 - 1861 K. HELIN ET AL. 'Heterodimerization of the transcription factor E2F-1 and DP-1 leads to cooperative trans-activation' see page 1858, left column, line 51 - page 1859, right column, paragraph 6; figure 8 -----</p>	1-18

Information on patent family members

PCT/GB 93/02227

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